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EDITORIAL

Microscopic colitis: A therapeutic challenge

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Abstract

The treatment of microscopic colitis is mainly based on the use of budesonide, the only drug found effective in controlled clinical trials. After an initial course at a dose of 9 mg daily, however, most patients relapse when the drug is discontinued, hence a maintenance therapy at doses of 6 mg daily or lower is necessary. In order to avoid steroid dependence and drug toxicity different pharmacological agents should be considered as an alternative to indefinite long-term budesonide treatment. Evidence-based guidelines are currently lacking due to the lack of conclusive data concerning the use of either immunosuppressive or anti-tumor necrosis factor agents. For the time being in clinical practice the skilled physician should therefore tailor long term management of microscopic colitis on the single patient.

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Key words: Microscopic colitis; Budesonide; Mesalazine; Immunosuppressants

Core tip: The efficacy of short-term treatment of microscopic colitis with budesonide is confirmed. Long-term therapy is not advisable because of possible side effects, but the efficacy of alternative drugs such as immunosuppressants or anti-tumor necrosis factor agents remains to be established. For the time being prolonged budesonide treatment in minimal doses, tailored on the single patient, appears to be the most sensible option.

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MICROSCOPIC COLITIS

Microscopic colitis (MC) is an intestinal inflammatory disorder the diagnosis of which relies on specific histopathological findings, namely an increased number of lymphocytes in the colonic epithelium and of subepithelial chronic inflammatory cells (lymphocytic colitis), in some cases with a tickening of the subepithelial layer (collagenous colitis)[1,2].

Radiographic and endoscopic features are constantly normal. The main symptom is chronic watery diarrhea without bleeding, the disease being more common among older individuals, especially of female gender. Although a genetic cause has not be proven, familial occurrence has been reported.

Smoking is a risk factor and bile acid malabsorption is frequent (however, a bile acid binding drug such as cholestyramine may improve symptoms but not histopathology)[1-3].

Medications such as nonsteroidal antiinflammatory drugs, proton pump inhibitor, ticlopidine, sertraline *etc.* can induce MC[4]. Hence, accurate information about pharmacological treatment history is mandatory in order to discontinue the supposedly responsible drug.

The only medication found effective in randomized, placebo-controlled trials is budesonide, which, at a dose of 9 mg per day is able to induce clinical remission and histological improvement in about 81% of cases[5]. The superior efficacy of budesonide compared with placebo has been shown in four controlled trials involving patients with either collagenous or lymphocytic form of MC[6-9].

By contrast only a small trial comparing prednisolone and placebo for two weeks reported a trend toward clinical response[10]. At any rate, budesonide should be preferred to other steroids not only because of fewer side effects, but also because the success rate is higher and the incidence of clinical relapses is lower[11,12].

When budesonide is withdrawn, symptomatic relapse of MC can occur in 60%-80% of cases[13,14]. In order to maintain remission budesonide can be successfully administered at a dose of 6 mg daily, up to six months[14,15]. After that period there is no published evidence that the drug continues to be effective, but clinical practice shows that budesonide 3-6 mg daily can prevent recurrence, although patients become at risk of becoming steroid dependent and to develop side effects due to long-term steroid therapy. The minimum dose of budesonide should be employed, even 3 mg every other day being occasionally sufficient to maintain clinical remission (Guslandi M, unpublished data), but in order to avoid steroid dependence and drug toxicity other therapeutic options must be considered.

Mesalazine, which is usually well tolerated, would represent an ideal long-term treatment, but evidence of its efficacy in MC is weak, retrospective series reporting benefit in fewer than half the patients as a short term therapy while data for periods exceeding 6 mo are lacking[16,17].

Immunosuppressive agents can be taken into consideration. both in patients with severe symptoms who do not respond to full doses of budesonide or who are experiencing side effects and/or steroid dependence during long-term budesonide treatment. Unfortunately available data with either azathioprine (or 6-mercaptopurine) and methotrexate in MC are extremely limited and inconclusive[18-20] despite their not infrequent use in clinical practice by gastroenterologists.

In the attempt to avoid colectomy in severe cases of MC refractory to any other pharmacological treatment, the possible use of biological agents has been tested, with promising results[21,22] but more conclusive data are needed.

Thus, long-term management of microscopic colitis remains elusive, especially in patients refractory or intolerant to budesonide, but even in subjects where the drug is effective but continuous intake for an indefinite length of time is not advisable. In those cases physicians must take therapeutic decisions irrespective of evidence-based data, tailoring the treatment on the characteristics and needs of the single patient. Even a recent treatment algorithm proposed by the European Microscopic Colitis Group[23] includes drugs such as loperamide, mesalazine, cholestyramine and bismuth, the efficacy of which is questionable and uncertain, pointing out the fact that the use of those medication is empirical. The same applies to immunosuppressants and anti-tumor necrosis factor agents, although the former, in spite of the scarce controlled data, appear to be a sensible and comparatively safe approach. Needless to say, randomized, controlled studies with azathioprine or methotrexate are eagerly awaited and sorely needed.

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REVIEW

Liver-spleen axis: Intersection between immunity, infections and metabolism

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Abstract

Spleen has been considered a neglected organ so far, even though is strictly linked to liver. The spleen plays an important role in the modulation of the immune system and in the maintenance of peripheral tolerance *via* the clearance of circulating apoptotic cells, the differentiation and activation of T and B cells and production of antibodies in the white pulp. Moreover, splenic macrophages are able to remove bacteria from the blood and protect from sepsis during systemic infections. We review the spleen function and its assessment in humans starting from the description of spleen diseases, ranging from the congenital asplenia to secondary hyposplenism. From the literature data it is clear that obesity in humans affects different compartments of immune system, even thought there are still few data available on the implicated mechamisms. The intent is to enable clinicians to evaluate the newly recognized role of metabolic and endocrine functions of the spleen with special emphasis to obesity and nonalcoholic fatty liver disease in the context of the available literature. Moreover, understanding the spleen function could be important to develop appropriate prevention strategies in order to counteract the *pandemia* of obesity. In this direction, we suggest spleen longitudinal diameter at ultrasonography, as simple, cheap and largely available tool, be used as new marker for assessing splenic function, in the context of the so-called liver-spleen axis.

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Key words: Spleen size; Obesity; Non-alcoholic fatty liver disease

Core tip: From the literature data it is clear that obesity in humans affects different compartments of immune system. The aim of this review is to let clinicians appreciate the new role of metabolic and endocrine functions of the spleen with special emphasis to obesity and nonalcoholic fatty liver disease in the context of the available literature. Moreover, understanding the spleen function could be important to develop appropriate prevention strategies in order to counteract the *pandemia* of obesity. In this direction, we suggest spleen longitudinal diameter at ultrasonography, as simple, cheap and largely available tool, be used as new marker for assessing the liver/spleen axis.

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INTRODUCTION

In vertebrate evolution, spleen functions were performed by a spleen-like tissue scattered along the digestive tract, as seen in lamprey. Bony fishes and sharks are the first

vertebrates where it appears as individual organ[1].

The spleen is a secondary peripheral lymphoid organ located in the abdominal cavity between the diaphragm and the fundus of the stomach of mammals. Its principal function was preserved during the evolution in all animal classes having that organ, while important differences can be observed histologically. For example, the red pulp is seen only from bony fishes upwards.

It is the largest lymphoid organ in the human body and it has a fundamental role as destruction of red blood cells and as actor in the immune response, filtering the blood from antigenic particles and from abnormal and aged cells. Table 1 summarizes the different functions of the spleen.

The spleen anatomical architecture is extremely sophisticated and little is still known about specific processes that are performed in its differentiation. Mesenchymal, hematopoietic and endothelial cells interact each other thanks to complex, organized and still undiscovered signals leading to the development of its complex micro-architecture[2-4].

Table 1 Function of the spleen

Red pulp
Extramedullary hematopoiesis if required
Facilitating an environment wherein erythrocytes rid themselves of solid waste material
Blood filter for foreign material and damaged and senescent blood cells
Storage site for iron, erythrocytes, platelets, plasmablasts and plasma cells
Rapid release of antigen-specific antibodies into the circulation produced by red pulp plasma cells
Defense against bacteria using iron metabolism by its macrophages
White pulp
T cell zone (periarterial lymphatic sheath) and B cell zone (follicles)
Storage site for B and T lymphocytes
Development of B and T lymphocytes upon antigenic challenge
Release of immunoglobulins upon antigenic challenge by B lymphocytes
Production of immune mediators involved in clearance of bacteria such as complement, opsonins, properdin and tuftsin
Marginal zone
Phagocytosis of circulating microorganisms and immune complexes by MZ macrophages
Development of marginal zone B lymphocytes upon TI-2 antigenic challenge
Blood trafficking of B and T lymphocytes
Release of immunoglobulins upon antigenic challenge by splenic B lymphocytes

WHAT EVIDENCE HAS SUGGESTED SPLEEN BE CONSIDERED A NEGLECTED ORGAN?

The congenital asplenia may occur with or without other clinically evident abnormalities. In the first case, with asplenia, other defects of organs of the thoracic and abdominal cavities can be found. One example is the heterotaxy syndrome, where there is a failure in the left-right axis specification[5].

If the defect occurs before the ontogenesis of the spleen on the left side, it may not affect splenic development. The second type of congenital asplenia is less common[6-10] and includes subjects with no other obvious abnormalities that report recurrent infections from childhood. In those cases the diagnosis of asplenia often remains unravelled, due to the lack of necroscopy.

Studies in mice have highlighted that some genes are crucial for spleen development, such as *Tcf21*, *Bapx1*, *Pbx1*[11] and recently also *Tlx1*[12]. In this case it can be expected that asplenic animals suffer from additional several anomalies caused by the deficiency of specific genes. However, in the literature are not reported corresponding cases, probably because in humans and mice similar genes do not have overlapping functions, or these subjects die before or soon after birth and/or they were not extensively investigated.

A suitable example may be the Atrx syndrome, where the mutations of this gene result in athalassemia, myelodysplasia and mental retardation[13]. Individuals with this syndrome occasionally exhibit asplenia[14], but the inactivation of Atrx similar gene in mice does not end up in asplenia[15].

In individuals with congenital isolated asplenia are reported some mutations[16], but the molecular mechanisms and the etiology of spleen development are still unknown.

ANATOMICAL AND HISTOLOGICAL COMPOSITION OF THE SPLEEN (ANIMAL *VS* HUMAN MODEL)

In the mice the spleen has a characteristic histological organization similar to a sponge, where the fibrous capsule form a reticular network with the trabeculae stemming from its internal side. The splenic artery enters the hilum of the spleen, divides itself into smaller branches and finally gives rise to "central arteriolae" of the white pulp and to the large sinusoids of the red pulp. The central arteriolae are surrounded by a sheath of small T lymphocytes, the so-called PeriArteriolar Lymphoid Sheath (PALS). They communicates with follicles, a highly organized accumulation of T and B lymphocytes. The red pulp, PALS and follicles are also surrounded by the marginal zone, filled with large memory B cells.

The human and mice spleen are not anatomically different. The fact that patients with autoimmune thrombocytopenia purpura and circulating antiplatelet antibodies improve after splenectomy[17], supports the role of the red pulp of the spleen in the displacement of old and damaged platelets, aged erythrocytes and apoptotic cells in humans.

After apoptosis of aged erythrocytes, hemoglobin is digested and iron is released or stored by splenic macrophages. Thus, the spleen is fundamental in the recycling of iron[18].

Interestinghly, after abdominal surgery for trauma or neoplasia, the displacement of the spleen is often without immediate consequences. This is one of the reasons for which, untill a recent past, the spleen was considered not a vital organ. Consequently, it was believed that the spleen could be removed without major consequences. Recently, Ozban *et al*[19] have disproved this theory, because they have shown that exercise in splenectomized individuals can cause serious problems in form of decreasing splanchnic flow and increasing blood viscosity.

The spleen plays an important role in the modulation of the immune system and in the maintenance of peripheral tolerance *via* the clearance of circulating apoptotic cells, the differentiation and activation of T and B cells and production of antibodies in the white pulp[20,21]. Moreover, splenic macrophages are able to remove bacteria from the blood and protect from sepsis during systemic infections.

Vice versa, most important differences between mice and humans in the spleen organization and functionality are revealed in the immune response. The marginal sinus in mice and the perifollicular zone in humans are areas of particular activity. B cells in the marginal zone in mouse are highly reactive and specialized against pathogens invasion *via* a T-independent reaction[20], however in humans, the same area contains memory B cells[22]. Several chemokines and adhesion molecules regulate the trafficking between the marginal zone and the white pulp.

ROLE OF SPLEEN IN LIMITING BACTERIAL INFECTION

As seen before, congenital asplenic subjects have an increased risk of developing infective diseases and the lack of the spleen functionality causes more startling effects in the newborn.

Morris *et al*[23] firstly described in 1919 that splenectomized patients are more susceptible to infections, especially caused by *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis* (so called encapsulated bacteria)[23-25]. The risk of sepsis is 10- to 20-fold higher than non splenectomized population and, especially in young children, death can result[26]. Overwhelming post-splenectomy infection can occur some hours after the first signs of deterioration of health and can degenerate to multi-organ failure and death[27]. Most asplenic children die of infection during the neonatal period. In fact, among the causes of sudden and unexpected infant death, the congenital asplenia can also be included[28].

Another condition is functional asplenia, when in patients with haematological or metabolic disorders the splenic tissue organization is altered and, for this reason, equally they develope the same type of infections. In patients with functional or anatomical asplenia is quite impossible to quantify the risk of developing infections and sepsis. Therefore, a method to conserve some splenic tissue during abdominal surgery with deracination of the spleen is to transplant small spleen fragments into the well vascularised greater omentum. Clinical data have shown that this procedure has an important effect in increasing specific antibody responses after pneumococcal vaccination, as well as normalizing IgM levels[29,30], and probably can also reduce the risk of opportunistic infections in immunodeficient subjects[31]. The increased susceptibility of hypo or asplenic individuals to encapsulated bacterial infections is mostly due to the lack of IgM memory B cells and to their not adherent reaction to polysaccharide vaccines. The absence of splenic macrophages with the reduced number of B cells in asplenic patients can result in the establishment of a favorable environment to the development of overwhelming bacterial infections.

SPLEEN AND NATURAL ANTIBODIES

A particular subtype of B-cell population is involved in the immune deficiency and in the reduced response to polysaccharide antigens seen in the asplenic or splenectomized mice. B cells may be divided in two main subpopulations on the basis of life development (fetal or adult), superficie markers and functions. Asplenic mice lack B-1a B cells[32], a distinct population from the more conventional B-2 B cells that are involved in the adaptive immunity and collaborating with T cells[33].

Functions of B-1a B cells are mainly three: (1) they can act in an T-cell independent mode during the immune response; (2) they produce natural antibodies and co-operate with the innate immune system to contrast bacterial and viral infections; and (3) in the intestinal mucosa they can differentiate into plasma cells producing IgA[34].

In the specific immune response, produced antibodies have a very high affinity for a particular epitope and they can prevent re-infection from the single pathogen that previously has caused their own production. *Vice versa,* the pentameric IgM isotype produced by B-1a B cells binds various antigens with high avidity and low affinity and is therefore able to neutralize many different antigens. IgM antibodies are the so called "natural antibodies" and in recent years, it has been demonstrated that they may play a role in the protection against malignancy[35] and atherosclerosis[36]. Asplenic mice not only have a reduced number of B-1a B cells but they have a decreased concentration of serum IgM[32].

B-1a B cells produce IgA immunoglobulins, and in the intestinal mucosa about half of the IgA plasma cells derives from B-1a B cells[37]. The homeostasis of the intestine is finely regulated by mucosal IgA. These immunoglobulins interact with antigens presented by the intestinal microbiota and by pathogens, preventing their overgrowth and subsequent invasion.

The precise reason why B-1a B cells are absent in asplenic mice and why their number rapidly declines after splenectomy is not yet defined. B-1a B cells are produced in the fetal liver, contrarily to B-2 B cells that derive from adult bone marrow. It is noteworthy to stress that the

first subtype cannot be replaced after adult bone marrow transplant. Moreover, Ig-positive precursors of B-1a B cells have been detected in the spleen, but it is unknown if these cells persist in the spleen during the adult life and derive from precursors situated in the fetal liver. According to a recent theory the spleen might be central to their generation or survival and therefore splenectomy would lead to the depletion of the B-1a population[32].

OTHER FUNCTIONS OF THE SPLEEN

An interesting hypothesis relates the spleen to the activity of gut-associated lymphoid tissue (GALT). The dysfunction of GALT is known to predispose to inflammatory bowel diseases (IBD), above all for its role in T cell activation and trafficking in the gut. Moreover, the frequency of IgM memory B cells is decreased in IBD subjects[38] establishing a relationship among GALT and spleen in humans.

The spleen also has important hematological functions. The spleen picks up from the circulation platelets that subsequently are stored or can be destroyed by lymphocytes. As storage organ the spleen stores about one third of the human body's platelets.

The thrombocytopaenia is a result of hypersplenism, because of the heightened functions of the spleen in sequestering and break-downing platelets. Conversely, after splenectomy mild thrombocytosis can be observed[39] because of the lack of sequestering and destruction of platelets by the spleen, and at the same time it can be observed a slight increase in platelet production in the bone marrow[40]. Erythrocytes are stored and removed from the blood circulation in this organ. After splenectomy, the presence in the blood of many substances released from circulating damaged erythrocytes with procoagulant activity can lead to the establishment of a procoagulant state and therefore to the occurrence of thromboembolic events (for example pulmonary embolism, deep vein and portal vein thrombosis).

Beyond haematopoietic stem cells, stem cells of other differentiation lines, such as stromal cells with osteogenic differentiation properties, seem to be present in the spleen, confirmed by *in vitro* studies[41]. It would be interesting to explore if in the spleen these osteogenic precursors may represent a monocyte/macrophage lineage common precursor cell population with the ability to differentiate along the osteoclast lineage[42]. Animal studies have also shown that splenocytes can differentiate into pancreatic islets and ductal epithelial cells when injected into diabetic non-obese diabetic mice, thereby splenocytes may be useful in the treatment of type 1 diabetes, thanks to their ability of restoring normal glycaemia[43,44]. Subsequently, Chong *et al*[45] have questioned the origin of these stem cells.

ASSESSMENT OF SPLEEN FUNCTION

Over the years, several methods have been developed to study the activity of the spleen. Because of its ability in purifying the blood from old erythrocytes, the amount of altered red blood cells can be used as index of functionality of the spleen. The detection of Howell-Jolly bodies is one of these, although the sensitivity and specificity are questionable for the hyposplenism[46,47]. Other haematological parameters are finding membranes pits, large vacuoles situated near to the plasma membrane, or other cellular changes as acanthocytes, target cells, Heinz bodies (remnants hemoglobin), Pappenheimer bodies (iron granulocytes) and siderocytes[48].

The count of B cells derived from the marginal zone, which have fundamental action in innate immunity, and in particular as defense against the invasion by encapsulated bacteria, is otherwise a possible method for evaluating the immunological activity of spleen[49]. B cells derived from the marginal zone and the memory cells producing IgM are in fact reduced in patients with diminished splenic function[38].

All the tests described above could be used with ease in clinical practice, on the other hand they have proved not to be very sensitive and specific, or they are needed to be further studied and validated[46,50].

To date, the radioisotope method is definitely the best way to quantify the filtering activity of the spleen. The (99m) Tc-labeled, heat-altered, autologous erythrocyte scintigraphy with multimodality single photon emission computed tomography (CT) - technology is considered the best approach to gauge all the facets of the splenic function[51]. However, this is a method that has the highest cost and it is difficult to perform.

SPLEEN AS A NEW PLAYER

Nonalcoholic Fatty Liver Disease (NAFLD), the most common cause of liver steatosis, is associated with obesity, mainly visceral type, and insulin resistance. The liver inflammation (nonalcoholic steatohepatitis, the so called NASH) can progress from the simple hepatic steatosis or fatty liver (FL) lasting risk factors, as type 2 diabetes mellitus, major obesity and Metabolic Syndrome (MS). In its natural history, NASH can end up in perisinusoidal fibrosis and cirrhosis. Hepatocytes, during steatosis, are fat-laden and swollen, and in steatohepatitis the hydropic change (ballooning) causes further swelling and also sinusoidal distortion, as visualized by *in vivo* microscopy studies. This evenience leads to the reduction of intrasinusoidal volume and microvascular blood flow, as clearly described by Farrell *et al*[52]. Sinusoidal endothelial cells, Kupffer cells and stellate cells are also involved in the pathological process in conjunction with the activation of the immune system. The microcirculation is skewed by inflammatory cells and platelets recruited in the liver. Animal models confirm these data and evidence that these pathological changes lead to a marked reduction of sinusoidal space (approximately 50% of control), and a decrease in the number of normally perfused sinusoids, according to a review, recently published[51]. The micro-

vascular damage is necessary for developing futher liver injury and causing disease progression as in NASH. The lipid peroxidation of unsaturated fatty acids by reactive oxygen species is one of the main causes of the sensitivity of hepatic steatosis to ischemia-reperfusion injury. During the whole 24-h-period the most part of time is spent in postprandial state in humans. Therefore, the liver has a fundamental role in maintaining the correct energy state balancing the input, secretion, and oxidation of fatty acids. In abdominally obese men the oxidation of dietary fatty acids, hepatic desaturation and elongation of palmitic acid occur to a greater extent than in non-obese[53].

This means, therefore, that donor's fatty livers are an obstacle to transplantation[52]. Between other hepatic cells, the dysfunction of Kupffer cells gives a major contribution to NASH progression. It is noteworthy that the reticular-endothelial system also plays a key role in the spleen and a good method for study Kupffer cell activity is the colloid scintigraphy. Duman *et al*[54] have followed 22 patients with biopsy-proven NASH who underwent colloid liver scintigraphy. Liver right/left lobe ratio was altered in all patients after intravenous injection of 185 MBq Tc tin colloid. The shift of colloid to the spleen and a prolonged blood pool clearance time was observed in 55% of patients with NASH.

Previously, Tsushima *et al*[55] aimed to determine if there was an association between spleen enlargement and NAFLD, measuring spleen volume at CT. It must to be observed that the values were weighted according to the patient's demographic data, the Liver/Spleen (L/S) ratio of CT Hounsfield unit measurements, and liver function tests. L/S ratio was also used to perform the diagnosis. The authors evidenced an increased mean spleen volume ($P < 0.0001$) between NAFLD and controls 73.0 ± 24.4 cm^3 (range, 21.1-106.1) in normal subjects and 141.2 ± 54.1 cm^3 (range, 44.1-267.3) in NAFLD subjects. Only the L/S ratio ($P < 0.0001$) and age ($P < 0.01$) were significantly correlated to spleen volume at multivariate linear regression analysis and at forward selection stepwise regression.

Basing on the evidence that obesity and insulin resistance are inflammatory chronic diseases and therefore are associated with systemic markers of inflammation, some scholars have attempted to find a non invasive diagnostic method for NASH to help clinicians to decide whether and when to perform liver biopsy.

Patients with histology proven-NAFLD (43 patients with NASH and 40 with FL), compared with healthy subjects, were evaluated with ultrasonographic exams, with particular interest to ultrasonographic spleen longitudinal diameter (SLD) and splenic artery resistive index, and laboratory measurements, as serum interleukin (IL)-6 and vascular endothelial growth factor (VEGF) concentrations. The NASH group demonstrated higher IL-6 blood levels, SLD values, and VEGF concentrations than controls. In this study was estimated that the SLD is more sensitive than IL-6 and VEGF in discriminating NASH from FL, and the optimal cut-off value for SLD is 116 mm (specificity 95% and sensitivity 88%). NASH and FL subjects have a similar splenic artery resistive index, but it differs when compared with controls. On the other hand, normal values of SLD and IL-6 were associated with FL and normal values of IL-6 could confirm the absence of NASH[56]. Further confirmation of these findings comes from another study which highlighted that spleen enlargement may be a distinct feature of NASH, especially early-stage NASH[57]. Therefore, we suggest that SLD could be used as new marker for assessing splenic function, independently from its use in distinguishing the simple FL, also called benign, from NASH, the more severe form of NAFLD, benignity not always shared[58].

In this study[59], SLD and blood pressure were significantly correlated with insulin resistance, moreover measures of SLD were well predicted by body mass index values.

To let Authors duplicate this finding, SLD was measured by postero-lateral scanning. It was used the average value obtained by measuring the maximum length and the cranio-caudal diameter. All the indices were measured thrice.

A subsequent study showed that spleen enlargement was found at significant levels (38%) in obese female rats as determined by Cavalieri volume calculation, an unbiased stereological method[60]. These recent results clearly indicated that high fat diet caused splenomegaly *via* sinusoidal dilatation and intracellular or intercellular deposits[61]. Although these data are encouraging to find a non-invasive method for NAFLD diagnosis, liver biopsy remains the only reliable method to differentiate simple steatosis or FL from NASH in NAFLD subjects[58]. On this line, Kikuchi *et al*[62] evaluate the efficacy of non-invasive (99m) Tc-phytate scintigraphy in the diagnosis of NASH in humans and in a rat model. In the first study, patients with suspected NAFLD underwent liver biopsy and (99m) Tc-phytate scintigraphy. As region of interest, signal intensities of the liver and spleen were measured. Subsequently, they observed that the L/S uptake ratio at scintigraphy was significantly decreased in NASH subjects when compared to patients with FL. The L/S ratio was an independent predictor in distinguishing NASH from FL. More interestingly, the decrease of L/S ratio was found in all NASH stages, from its earliest stages (stages 1 and 0). In the second study, the authors induced NASH in rats feeding them with a Methionine- and Choline-Deficient (MCD) diet. In this case, the L/S uptake ratio was also significantly decreased after 8 wk of a MCD diet in comparison with control diet-fed rats. From these data, the authors concluded that non-invasive (99m) Tc-phytate scintigraphy is able to discriminate NASH from FL.

INFECTIONS TENDENCY IN OBESITY AND THE POSSIBLE LINK WITH THE SPLEEN

The frequency of ischemic heart disease observed after traumatic splenectomy and the low cholesterol levels

Table 2 Main topics

Congenital asplenia in humans
There are two types of congenital asplenia: with or without other clinically evident abnormalities
Tcf21, Bapx1, Pbx1 and Tlx1 are crucial for spleen development
The molecular mechanisms and the etiology of spleen development are still unknown
How the anatomical and histological composition of the spleen can guarantee its function?
The phagocytosis of old and damaged cells, particles and blood-borne microorganisms from local macrophages takes place in the red pulp
The spleen is fundamental in the recycling of iron
Exercise in splenectomized individuals can decrease splanchnic flow and increase blood viscosity
Most important differences between mice and humans in the spleen organization and functionality are revealed in the immune response
Role of spleen in limiting bacterial infection
Splenectomized and asplenic patients are more susceptible to infections, especially caused by Haemophilus influenzae
Subjects with functional asplenia develope the same type of infections
The spleen and natural antibodies
B cells may be divided in two main subpopulations on the basis of life development (fetal or adult), superficie markers and functions
Spleen might be central to the generation or survival of the B-1a population and therefore splenectomy would lead to their depletion
Other functions of the spleen
There is a probable relationship among GALT and spleen in humans
The spleen also has important hematological functions
In the spleen were found stem cells with several differentiation properties: haematological, osteogenic and maybe pancreatic
Assessment of spleen function
Hematologycal and immunological parameters should be used in the assessment of spleen function
The best approach to gauge all the facets of the splenic function is the radioisotope method
Spleen as a new player
There is an association between spleen enlargement and NAFLD
SLD could be used as new marker for assessing splenic function
Initial data have shown that SLD is more sensitive than IL-6 and VEGF in discriminating NASH from FL, and the optimal cut-off value for SLD is 116 mm
Infections tendency in obesity and the possible link with the spleen
Obese subjects have an increased risk to develop malignancies and infections
The pathophysiological mechanisms by which cellular immune functions are affected by obesity are still under investigation but the spleen may have an important role

GALT: Gut-associated lymphoid tissue; NAFLD: Nonalcoholic fatty liver disease; SLD: Spleen longitudinal diameter; VEGF: Vascular endothelial growth factor; IL-6: Interleukin-6; NASH: Nonalcoholic steatohepatitis.

found in patients with hypersplenism are observations that suggest a possible role for the spleen in lipid metabolism and in the etiology of atherosclerosis[63]. Previous studies showed that obese subjects, compared to non-obese, have an increased risk to develop cardiovascular disease, hypertension, cerebrovascular disease and type 2 diabetes mellitus. But, it is equally important that they have an impaired immune function, as demonstrated by the higher incidence of malignancies and infections. From the literature data it is clear that obesity in humans affects different compartments of immune system, even thought there are still few data available on the implicated mechamisms. Elderly people (> 60 years of age) have an increased risk of infection, showing their peripheral blood lymphocytes a decreased reactivity to mitogens and an impaired proliferative capacity[64,65]. The response of T lymphocytes to concanavalin A and response of B lymphocytes to pokeweed mitogen are decreased in obese subjects[66]. In addition to the T lymphocyte population, also natural killer cell activity is suppressed in obese men and women > 60 years of age, as mentioned in a report made by Moriguchi *et al*[66,67]. Moreover, the natural killer cells activity and percentage of body fat are negatively correlated in both elderly women[68], and middle-aged men[69]. These data suggest that obesity is a risk factor for the progressive deteriorating of cellular immune functions. The pathophysiological mechanisms by which cellular immune functions are affected by obesity are still under investigation but the spleen may have an important role. In the splenic lymphocytes of obese mice, the expression of glucose transporter 1 (GLUT-1), analyzed by Western blot analysis, was lower compared to lean rats. The decreased expression of GLUT-1 in these rats is associated with a defective uptake of glucose into immune cells. It is probable that the decreased proliferation of splenic lymphocytes in obese rats is connected to the decreased expression of GLUT-1 and therefore to an impairment of glucose uptake[69]. An interesting report by Miyake *et al*[70] evaluates NAsFLD mice feed high-fat and high-calorie diet for 12 wk for assessing the extent of antigen-specific immunity response. NAFLD mice and control mice were immunized with hepatitis B vaccine containing hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) and, subsequently, antibody to HBsAg (anti-HBs) blood levels, HBsAg and HBcAg-specific cellular immune response and functions of whole spleen cells, T lymphocytes, B lymphocytes and spleen dendritic cells (DCs) of NAFLD and control mice were assessed *in vitro*. Interestingly, in NAFLD mice levels of anti-HBs and the proliferation activity of HBsAg and HBcAg-specific lymphocytes were significantly lower compared to controls. Higher levels of inflammatory cytokines were produced and T cells have showed an increased proliferation rate in spleen cells of NAFLD than lean mice. Concurrently, DCs processing and presenting antigen activities were significantly decreased in the spleen of NAFLD mice compared to controls. Moreover, the administration of saturated fatty acids caused impaired antigen processing and presenting capacity of murine DCs. These data emphasize that the modification of antigen-specific immunity in NAFLD mice depends on the action different types of immunocytes, including DCs and lymphocytes, clarifying the role of the spleen in this specific pathological process.

FUTURE PERSPECTIVES

This paper reports the studies on the use of simpler parameters in assessing the need for medical intervention with respect to healthy and non healthy overweight/obese individuals.

In a not too distant past, the spleen has been considered a neglected and expendable organ. In portal hypertension it was considered an ancillary organ[71-74] and it has some importance in infectious disease or as organ localization in lymphoproliferative diseases.

As described before, Table 2, now the spleen is deemed an important component of the immune system, crucial in immune response regulation[55,75,76], and also it has a metabolic asset and it is involved in endocrine function with regard to NAFLD[51].

It is suggested that adoption of a simpler tool to perform measurements could not only reduce the cost of medical care but also provide more reliable identification of patients in need of weight loss[77-80].

Larger and well-implanted studies comprehending better characterized patients should be taken into account to ascertain the validity of this tool.

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REVIEW

Evaluation of hepatic cystic lesions

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Abstract

Hepatic cysts are increasingly found as a mere coincidence on abdominal imaging techniques, such as ultrasonography (USG), computed tomography (CT) and magnetic resonance imaging (MRI). These cysts often present a diagnostic challenge. Therefore, we performed a review of the recent literature and developed an evidence-based diagnostic algorithm to guide clinicians in characterising these lesions. Simple cysts are the most common cystic liver disease, and diagnosis is based on typical USG characteristics. Serodiagnostic tests and microbubble contrast-enhanced ultrasound (CEUS) are invaluable in differentiating complicated cysts, echinococcosis and cystadenoma/cystadenocarcinoma when USG, CT and MRI show ambiguous findings. Therefore, serodiagnostic tests and CEUS reduce the need for invasive procedures. Polycystic liver disease (PLD) is arbitrarily defined as the presence of > 20 liver cysts and can present as two distinct genetic disorders: autosomal dominant polycystic kidney disease (ADPKD) and autosomal dominant polycystic liver disease (PCLD). Although genetic testing for ADPKD and PCLD is possible, it is rarely performed because it does not affect the therapeutic management of PLD. USG screening of the liver and both kidneys combined with extensive family history taking are the cornerstone of diagnostic decision making in PLD. In conclusion, an amalgamation of these recent advances results in a diagnostic algorithm that facilitates evidence-based clinical decision making.

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Key words: Coincidental hepatic cystic lesions; Cystic liver disease; Complicated cyst; Polycystic liver disease; Diagnostic algorithm

Core tip: We performed a review of the recent literature, and through combining current consensus and recent advances, we developed an evidence-based diagnostic algorithm to guide clinicians in characterising hepatic cystic lesions. Serodiagnostic tests and microbubble contrast-enhanced ultrasound (CEUS) are invaluable in differentiating complicated cysts, echinococcosis and cystadenoma/cystadenocarcinoma when ultrasonography (USG), computed tomography and magnetic resonance imaging show ambiguous findings. As a result, serodiagnostic tests and CEUS reduce the need for invasive procedures. USG screening of the liver and both kidneys combined with extensive family history taking remains the cornerstone of diagnostic decision making in polycystic liver disease.

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INTRODUCTION

Hepatic cystic lesions represent a comprehensive heterogeneous cluster with regard to pathogenesis, clinical presentation, diagnostic findings and therapeutic management (Table 1). Hepatic cystic lesions predominantly remain asymptomatic and are found as a mere coinci-

dence on abdominal imaging techniques, such as ultrasonography (USG), computed tomography (CT) and magnetic resonance imaging (MRI)[1,2]. The use of these techniques has greatly increased over the last years, and as a corollary, there has been an increase in incidental findings of asymptomatic hepatic cystic lesions[3]. In most cases, hepatic cystic lesions will follow a benign course[4]. However, it is essential to differentiate benign cysts from potentially harmful cysts, such as echinococcosis, cystadenoma and cystadenocarcinoma, which require specific treatment[5,6]. Currently, clinicians must also be aware of changes in the epidemiology of certain hepatic cystic lesions. Echinococcosis has spread to previously non-endemic Western European countries[7,8]. For this reason, the early and accurate diagnosis of cysts is crucial. To facilitate the diagnostic process, we provide an overview of the wide spectrum of mono- and polycystic liver diseases based on literature published over the last five years.

LITERATURE SEARCH

We searched the electronic database PubMed using the following search terms: "liver" and "cyst" and "diagnosis". We limited our search to articles that were written in English, published between November 2007 and November 2012 and available in full text. A total of 992 articles were identified. For the purpose of this review, we included articles with a main focus on the evaluation of hepatic cystic lesions in humans. Screening the titles and abstracts identified 252 articles meeting these inclusion criteria (Figure 1). Additionally, we searched the reference lists from all eligible reviews for additional leads.

SIMPLE CYSTS

Pathogenesis

Simple cysts arise congenitally from aberrant bile duct cells and contain a clear, bile-like fluid[9]. Because bile duct epithelium covers the simple cyst inner lining, it is hypothesised that simple cysts arise during embryogenesis when intrahepatic ductules fail to connect with extrahepatic ducts[4,10].

Clinical features

The prevalence of simple cysts ranges from 2.5% to 18% and increases with age[11,12]. More than half of individuals older than 60 years are likely to have one or more simple cysts. Cysts are small in most patients but can grow to over 30 cm in selected cases. In a small fraction of patients, symptoms, such as abdominal pain, early satiety, nausea and vomiting, arise as a result of a mass effect[3]. Physical examination may reveal a palpable abdominal mass or hepatomegaly[1]. Complications such as haemorrhage, rupture and biliary obstruction are uncommon but are more likely in larger cysts[13]. Intracystic haemorrhage is a rare complication of simple cysts and usually presents with severe abdominal pain[14], although asymptomatic presentations are also observed[15,16].

Table 1 Differential diagnosis of cystic lesions in the liver

Monocytic disease
Simple cyst
Echinococcosis
Cystic echinococcosis
Alveolar echinococcosis
Cystadenoma
Cystadenocarcinoma
Polycystic disease
Autosomal dominant polycystic kidney disease
Autosomal dominant polycystic liver disease

Laboratory findings

Laboratory findings are predominantly normal, but a minority of patients have raised serum γ-glutamyltransferase (γGT)[17]. Several studies have shown that serum and cyst fluid levels of carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA 19-9) may be elevated[18]. CA 19-9 is expressed in the simple cyst inner epithelial lining and leads to elevated cyst fluid and serum CA 19-9 levels[17]. CA 19-9 is not helpful in the differential diagnosis of intracystic haemorrhage[19].

Diagnostic features

Most simple cysts are diagnosed incidentally on USG (Figure 2A), CT (Figure 2B) or MRI. The diagnosis of a simple cyst is based on the following USG criteria: anechoic (*i.e.*, fluid filled cavity), no septations, sharp smooth borders, strong posterior wall echoes (indicating a well-defined fluid/tissue interface), spherical or oval shaped and a relative accentuation of echoes beyond the cyst compared to echoes at a similar depth transmitted through normal adjacent hepatic tissue (Table 2)[20]. CT shows a sharply defined homogeneous hypodense lesion (Figure 2B)[21]. MRI T1-weighted sequence shows low signal intensity, whereas the T2-weighted sequence shows extremely high signal intensity, which does not enhance after contrast injection[22]. USG has a reported sensitivity and specificity of approximately 90% for diagnosing a simple cyst[23], and recent advances in CT and MRI technology might result in even higher sensitivity rates[12,22,24]. However, because CT is accompanied with a radiation load and both CT and MRI come at a significantly higher cost, USG remains the most accurate, non-invasive and cost-effective imaging modality for diagnosing simple cysts.

In case of an intracystic haemorrhage (*i.e.*, complicated cyst), USG typically shows a hyperechogenic echo pattern combined with internal echoes that mimic septations or solid portions (Figure 3)[25]. In contrast, CT visualises intracystic haemorrhage as a high-density area[26], whereas MRI depicts it as a high signal intensity on T1- and T2-weighted sequences[27]. Neither CT nor MRI has additional diagnostic value compared to USG in the diagnosis of cystic bleeding[15]. The recent development of microbubble contrast-enhanced ultrasound (CEUS) enables us to visualise vascular flow within septa or solid components of cysts, which is absent in simple cysts with intracystic

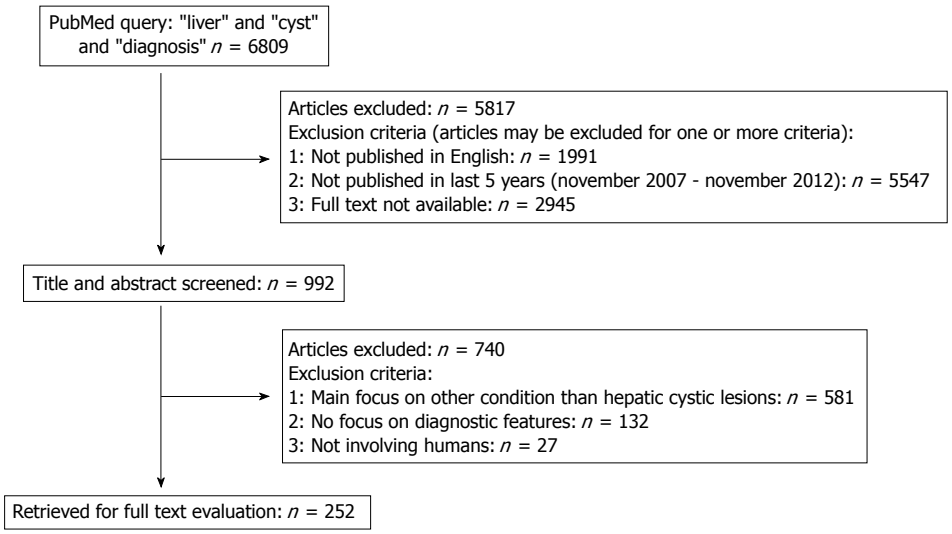

Figure 1 Selection process of retrieved articles.

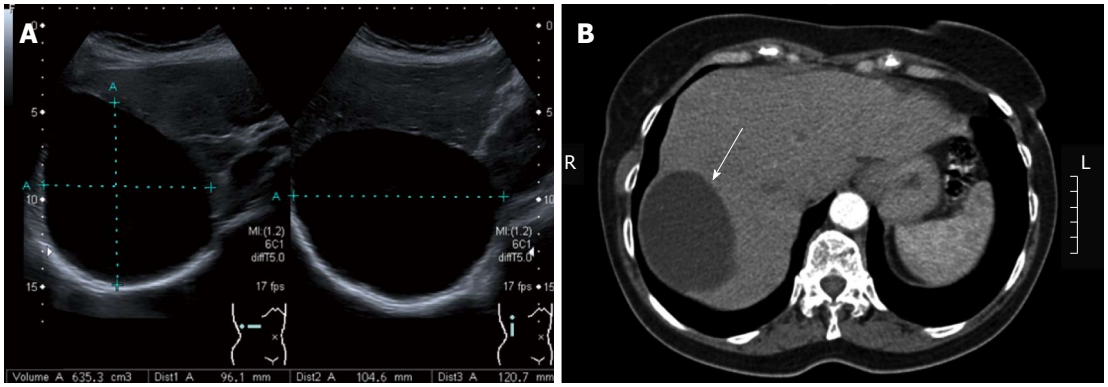

Figure 2 Simple cyst. A: On abdominal ultrasonography. Ultrasonography (USG) demonstrating a large simple cyst occupying the right hepatic lobe. Note the sharp and smooth border, oval shape, and anechoic echo pattern with the absence of septations and strong posterior wall echoes. The cyst size is indicated by the dotted lines; B: On abdominal computed tomography. Computed tomography demonstrating a sharply defined homogeneous hypodense cystic lesion (arrow) occupying the right hepatic lobe, which was diagnosed as a simple cyst.

haemorrhage[28]. Therefore, CEUS can accurately characterise these cysts when USG, CT and MRI show ambiguous findings[29-31].

Therapy

The management of most simple cysts relies on a "wait-and-see" policy, and no further treatment is required in these cases. If there are symptoms, aspiration-sclerotherapy is the preferred treatment[32,33]. Laparoscopic or open surgical fenestration techniques are similarly or even more effective in reducing symptoms[34,35] but have a significantly higher morbidity and mortality rate[36].

ECHINOCOCCOSIS

Echinococcosis is a zoonosis caused by larval stages of taeniid cestodes (tapeworms) belonging to the *Echinococcus* species. Two of the six known species cause solitary cystic lesions in humans: (1) *Echinococcus granulosus* (*E. granulosus*), responsible for cystic echinococcosis (CE); and (2) *Echinococcus multilocularis* (*E. multilocularis*), responsible for alveolar echinococcosis (AE)[6].

Echinococcosis-related deaths are uncommon in developed countries. For example, there were 41 echinococcosis-associated deaths in the United States over an 18-year study period[37]. However, echinococcosis is considered to be an emerging disease in Europe[38,39]. Thus, CE and AE are diseases with a considerable global disease impact, as indicated by a substantial loss in disability-adjusted life years[38,40].

Cystic echinococcosis

Pathogenesis: Humans become infected by acting as intermediate hosts of *E. granulosus* after ingestion of *Echinococcus* eggs, which are excreted by infected carnivores (dogs and other canids)[6]. Infection is typically observed in areas containing large numbers of the intermediate hosts of the parasite (sheep and goats) that are in close contact with the final host (herding dogs)[41-43].

Clinical features: Although CE has a worldwide geographic distribution, the highest prevalence of CE is

Table 2 Ultrasonography features for the diagnosis of monocytic diseases of the liver

	Simple cyst	Cystic echinococcosis	Alveolar echinococcosis	Cystadenoma and cystadenocarcinoma
Border	Sharp and smooth	Laminated	Irregular	Irregular
Shape	Spherical or oval	Round or oval	Irregular	Round or oval
Echo pattern	Anechoic[1]	Anechoic or atypical[2]	Hyperechogenic outer ring and hypoechogenic centre	Hypoechogenic with hyperechogenic septations
Appearance	No septa	Multiseptated	Multivesicular	Septated and/or solid structures (papillary projections)
Wall	Strong posterior wall echoes			Wall enhancement
Posterior acoustic feature	Relative[3] accentuation of echoes		Dorsal shadowing (calcified areas)	Dorsal shadowing (calcified areas)

[1]Fluid-filled cavity; [2]Snowflake-like inclusions or floating laminated membranes; [3]Compared to echoes at a similar depth transmitted through normal adjacent hepatic tissue.

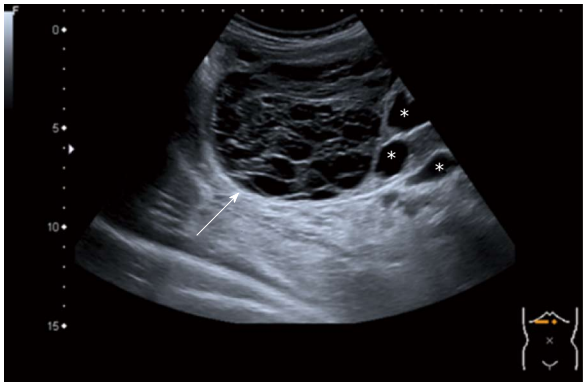

Figure 3 Complicated simple cyst on abdominal ultrasonography. Ultrasonography (USG) demonstrating a cystic lesion with a hyperechogenic echo pattern combined with internal echoes that mimic septations or solid portions (arrow) in a patient presenting with severe abdominal pain with a known history of multiple simple cysts (asterisks). Because of the known history of simple cysts, the lesion was diagnosed as a complicated simple cyst (*i.e.*, intracystic haemorrhage).

found in the temperate zones, including the Mediterranean, Central Asia, Australia and some parts of America[44].

Because cyst growth in the liver is slow (ranging from 1-5 millimetres in diameter per year), CE can remain asymptomatic for a long time. In approximately 90% of cases, the primary presentation is a spherical, fluid-filled vesicle with an inner cellular layer and an outer laminated layer located in the liver, lungs or both[45]. Symptoms occur when cysts exert mass effects within the organ or surrounding tissues or rupture, often presenting as a sudden onset of abdominal pain. Secondary cholangitis (rupture into the biliary tree), biliary obstruction and intraperitoneal rupture followed by anaphylaxis are common complications of CE and require hospitalisation[6]. Worldwide mortality rate estimates vary between 2.2%-5.0%[45,46], although the exact mortality rate of CE in developed countries remains unknown.

Diagnostic features: The diagnosis of CE is based on the following criteria: endemic region history, clinical findings (*e.g.*, abdominal pain, fever, chest pain, and dyspnea), pathognomonic USG features and positive immuno diagnostic tests[47]. USG shows a round or oval-shaped, anechoic or atypical (*i.e.*, snowflake-like inclusions or floating laminated membranes) echo pattern with multiple septa confined by a laminated border (Table 2)[47]. USG has a reported specificity of 90% and is used in combination with CT when surgical treatment is considered. MRI has not been proven to be cost-effective and has no added value[48]. The currently used serodiagnostic tests to reveal *E. granulosus* antibodies have a sensitivity of 93.5% and specificity of 89.7%[49].

Therapy: The treatment of CE, including surgery (open or laparoscopic), percutaneous treatments [*e.g.*, puncture aspiration injection re-aspiration (PAIR) method] and chemotherapy[50], is indicated to reduce symptoms and prevent complications[51]. PAIR is the treatment of choice for CE, as a recent review showed that PAIR resulted in parasitological clearance (*i.e.*, negative serodiagnostic tests) in 95.8% of cases[52].

Alveolar echinococcosis

Pathogenesis: AE is endemic in the Northern hemisphere (*e.g.*, North America, Asia, China, Japan and Europe). AE occurs when *E. multilocularis* eggs, found in the excrement of foxes, are ingested. The spread from endemic areas to previously non-endemic Western European countries is most likely due to an increasing fox population and spillover from these wild carnivores to domestic hosts[7,8].

Clinical features: The ingested eggs develop into an alveolar structure composed of numerous small vesicles that vary in diameter from smaller than 1 mm to 3 cm. Each vesicle has the same wall structure as CE. These vesicles grow slowly and are able to reach a maximum diameter of 15-20 cm, similar to simple cysts[53]. In approximately 99% of cases, the infection is initially confined to a solitary alveolar lesion in the liver[45]. After the primary infection, AE usually has an asymptomatic phase of 5-15 years prior to the development of symptoms. Symptoms are related to mass effect or are nonspecific, such as weight loss or fatigue[54]. In contrast to the encapsulated growth pattern of CE, AE eventually leads to liver failure

due to an infiltrative neoplastic growth with potential metastasis to adjacent and distant (*e.g.*, lungs, spleen, bone, and brain) organs[55,56].

Diagnostic features: Typical USG aspects are observed in 70% of cases and include irregular shape and border, hyperechogenic outer ring and hypoechogenic centre, multivesicular appearance and dorsal shadowing due to calcified areas (Table 2)[47]. Atypical USG aspects include small hyperechogenic nodules (amorphous AE), large lesions with massive necrosis (pseudocyst) and small calcified lesions (inert AE)[57]. In contrast to CE, MRI is superior to CT in detecting AE lesion margins[58,59]. Similar to CE, high diagnostic sensitivity (90%-100%) and specificity (95%-100%) are attained with serodiagnostic tests, and in 80%-95% of cases, AE can be differentiated from CE with the help of serologically obtained purified *Echinococcus* antigens[60].

Therapy: The approach to the management of AE resembles that of a hepatic malignancy. The cornerstone of treatment for AE includes radical surgery followed by a 2-year period of chemotherapy[6]. A recent study concluded that AE can be cured in 42% of cases by complete surgical removal of the parasitic mass. Early diagnosis could even improve this rate further[61].

CYSTADENOMA AND CYSTADENOCARCINOMA

Pathogenesis

Cystadenoma and cystadenocarcinoma are biliary cyst tumours that originate from the biliary epithelium[62]. Analogous to simple cysts, cystadenoma is considered to be a congenital disorder[63]. The exact mechanism of carcinogenesis in cystadenoma remains unknown. Several studies have suggested that cystadenocarcinoma develops from the ectopic remnants of primitive foregut sequestered within the liver[63]. In contrast, the malignant transformation of cystadenoma into cystadenocarcinoma is considered to be an alternative mechanism of carcinogenesis, as some cystadenocarcinomas may co-exist with cystadenoma[64]. This hypothesis is supported by the observation that the presence of cystadenoma increases the chance of developing cystadenocarcinoma[65].

Clinical features

Less than 5% of all cystic lesions of the liver are cystic neoplasms[2]. The clinical presentation of cystadenoma and cystadenocarcinoma is asymptomatic or tends to mimic symptoms of simple cysts or echinococcosis[66,67]. Studies have reported a predominance in women, with a mean age of onset varying from 40-60 years[64,65]. Cystadenomas appear to be slow growing, but exact growth rates are unknown. One case series evaluated 75 patients and recorded a variability in cyst size from 1.5-35 cm[68]. One study involving 63 cases diagnosed with cystadenocarcinoma demonstrated infiltrative growth in neighbouring organs in 33 cases (52%) and distant metastases in 15 cases (24%)[5]. For that reason, it is necessary to diagnose cystadenocarcinoma in an early stage.

Laboratory findings

In general, liver function tests are normal. A review of 13 cases found that serum concentrations of γGT and alkaline phosphatase (AP) were elevated in 3 cases[69]. One study reported a rise in serum levels of CEA in 3 of 22 cystadenocarcinoma cases (14%) and a rise in the serum concentration of CA 19-9 in 4 of 11 cases (36%)[5]. Similar results have been reported in cases with cystadenoma: one study showed elevated serum concentrations of CEA or CA 19-9 in 2 of 3 cases[63]. Consequently, laboratory studies are not helpful in differentiating cystadenoma and cystadenocarcinoma from complicated cysts or echinococcosis.

Diagnostic features

The USG characteristics of cystic neoplasms for both cystadenoma and cystadenocarcinoma are the following: a round or oval shape, irregular border, hypoechogenic echo pattern with hyperechogenic septations or solid structures (*i.e.*, papillary projections), wall enhancement and dorsal shadowing due to calcified areas (Table 2)[2]. Because of these typical cystic neoplasmic features, which are absent in simple cysts, USG is a useful technique to easily discriminate between cystic neoplasms and simple cysts[2]. Like USG, CT and MRI show markedly similar characteristics for cystadenoma and cystadenocarcinoma: internal septations, thickened and irregular wall, papillary projections, calcifications and wall enhancements[62]. Cystadenomas predominantly have thinner septa and more regular walls[70], whereas solid structures, intracystic haemorrhage and vascularised septations on contrast-enhanced CT are more suspicious for cystadenocarcinoma[62]. However, in most cases, differentiation between cystadenoma and cystadenocarcinoma is not possible[1]. The same problem arises in differentiating echinococcosis and complicated cysts from cystadenoma and cystadenocarcinoma because in many cases, intracystic haemorrhage, calcifications and septations are present in these lesions[2,62].

Recent advances in technology have made diffusion-weighted magnetic resonance imaging (DWI) a promising MRI technique for liver lesion detection and characterisation[71]. DWI depicts the rate of diffusion of water molecules between tissues, given as the apparent diffusion coefficient (ADC)[72]. Generally, high ADC values are measured in cystic and necrotic tissue, which allow a relatively free diffusion of water, whereas low ADC values are an indication of cell-rich tissue (*e.g.*, tumour tissue)[22,73,74]. However, because of an overlap of ADC values, differentiating cystic neoplasms, echinococcosis and complicated cysts is not possible with DWI[75]. Therefore, additional immunodiagnostic tests are needed to rule out echinococcosis. Fine needle aspiration (FNA) could be of additional help to exclude complicated cysts[76]; however, due to the risk of malignancy, FNA is generally

not performed. In contrast, CEUS can be helpful in differentiating cystadenoma and cystadenocarcinoma from complicated cysts when USG, CT or MRI is inconclusive. CEUS characterises the vascular flow within septa in cystadenoma and cystadenocarcinoma, which is absent in complicated cysts[29-31]. Nonetheless, surgical resection remains the golden standard for diagnosing cystadenoma and cystadenocarcinoma when CEUS is not available.

Therapy

The primary treatment of cystadenoma and cystadenocarcinoma is hepatic resection. A study in which 66 cases of cystadenocarcinoma were subjected to hepatic resection described a 3-year survival rate of 74%[5].

PCLD AND ADPKD

Polycystic liver disease

Polycystic liver disease (PLD) is arbitrarily defined as the presence of > 20 liver cysts[77]. Autosomal dominant polycystic liver disease (PCLD) and autosomal dominant polycystic kidney disease (ADPKD) are two distinct genetic disorders associated with the development of polycystic livers[78]. Liver function, as judged by parameters of liver synthesis, is not affected in PLD, as functional hepatic tissue remains unaffected[77,79].

Pathogenesis

During embryogenesis, the intrahepatic bile ducts are formed from a cylindrical layer of cells (*i.e.*, ductal plate) surrounding each portal vein. Incorrect involution of the ductal plate results in ductal plate malformation (DPM)[80,81]. DPM consists of excess embryonic bile duct structures in a ductal plate configuration that does not communicate with the normally developed intrahepatic bile ducts. The progressive dilatation of these excess intrahepatic structures during life results in multiple liver cysts[82]. Similar to simple cysts, these cysts contain a clear, bile-like fluid and an inner lining of cholangiocytes[83].

Genetics

PCLD was historically considered a phenotypic variant of ADPKD[84]. However, the presence of PLD in the absence of renal cysts led to the belief that PCLD should be regarded as a separate entity[85]. The discovery of a familial form of PLD[86], genetically distinct from the heterozygous mutation in genes *PKD1* and *PKD2* identified in ADPKD[87], ultimately led to the identification of heterozygous mutations in genes encoding *SEC63* and *PRKCSH*[88-90]. Mutation analysis identified a heterozygous mutation in *PRKCSH* (15%) and *SEC63* (5%) in approximately 20% of studied PCLD cases[91]. In contrast, a *PKD1* mutation was found in 85% of cases of ADPKD, and a *PKD2* mutation was found in the remaining cases[92].

PRKCSH and *SEC63* encode hepatocystin and SEC63 proteins, respectively. Hepatocystin acts in the folding process of proteins, while SEC63 acts as part of the endoplasmic reticulum translocon[93]. Unfortunately, the exact mechanism of cystogenesis in PCLD remains unclear. Polycystin 1 and 2, encoded by *PKD1* and *PKD2,* respectively, are important for adequate functioning of the primary cilium[94]. Its therefore suggested that primary cilia play a central pathogenic role in the mechanism of hepatic cystogenesis in ADPKD[78].

Clinical features

The extra polarisation of 137 identified PCLD cases in a specific adherence region (the Netherlands) led to an estimated PCLD prevalence of 1 per 158000[77]. This number is most likely an underestimation of the true prevalence because only symptomatic patients referred to tertiary centres were included in this study, and PCLD often remains asymptomatic[95]. ADPKD is the most common monogenetic disorder, with a world-wide estimated prevalence of 0.10%-0.25%[96], and it is responsible for approximately 8%-10% of cases with end-stage renal disease[97]. Although ADPKD is primarily characterised by the presence of renal cysts[98], liver cysts are considered the most prevalent extra-renal manifestation of ADPKD[99,100]. Indeed, one study involving 230 ADPKD cases found an overall prevalence of 83%[101]. However, the exact prevalence of PLD in ADPKD is still unknown. PCLD is predominantly confined to the liver, but a few renal cysts can also be present, which leads to difficulties in the accurate differentiation between PCLD and ADPKD[79,99]. Although renal cysts in ADPKD ultimately lead to renal failure, renal function remains unaffected in the presence of PCLD-associated renal cysts[102].

PLD is predominantly discovered during the fourth or fifth decade of life and is more severe in females[77,96,103,104]. PCLD tends to lead to a higher number and greater volume of liver cysts[79]. The number of pregnancies, increased age and severity of renal disease are considered additional risk factors for liver cyst growth in ADPKD[105]. PLD is mainly asymptomatic, but mechanical complaints can arise in a subset of patients[79,106]. Complications such as intracystic haemorrhage and infection are rare and typically occur in large cysts[106].

Laboratory findings

PLD causes increased γGT and AP levels in both PCLD and ADPKD patients[77]. Occasionally, increased serum aspartate aminotransferase (AST) is also found in ADPKD[79,107]. Renal function remains intact in PCLD, whereas ADPKD patients show a rise in serum creatinine due to impaired renal function[102].

Diagnostic features

PLD is detected with the use of USG, CT or MRI. USG, which is accurate, non-invasive and low cost, is the preferred imaging modality for both PCLD and ADPKD[108,109]. Currently, there are no generally accepted USG criteria for PCLD. One study suggested that the diagnosis can be made in case of a positive family history of PCLD and the presence of > 4 liver cysts[78]. However,

Table 3 Ultrasonography criteria for the diagnosis of autosomal dominant polycystic kidney disease

Family history positive[1]	
Unknown genotype	
Age (yr)	
≥ 15 and ≤ 39	≥ 3 unilateral renal cysts
≥ 40 and ≤ 59	≥ 2 bilateral renal cysts
≥ 60	≥ 4 bilateral renal cysts
Family history negative	
> 10 bilateral renal cysts, with the exclusion of renal or extra-renal disease causing renal cysts	

[1]Exclude autosomal dominant polycystic kidney disease when < 2 unilateral renal cysts and ≥ 40 years of age.

diagnosing ADPKD is usually relatively straightforward when enlarged bilateral cystic kidneys are present in combination with a positive family history for ADPKD[102]. In case of a negative family history, screening direct family members with USG can be helpful to reveal asymptomatic ADPKD. Because mutation analysis for ADPKD has no clinical implications, its use is limited to family members of ADPKD patients involved in kidney donation programs. In 2009, the Pei USG criteria were developed because the original Ravine USG criteria for diagnosing ADPKD appeared to be insufficient[110,111]. Table 3 gives an overview of the USG criteria for diagnosing ADPKD when the causative gene is unknown. For example, in case of a positive ADPKD family history, diagnosis can be made when ≥ 3 renal cysts are unilaterally present in individuals aged 15 to 39 years[110]. ADPKD should be considered when there are > 10 bilateral renal cysts present in the absence of other renal or extra-renal disease that can cause renal cysts[108]. When PCLD or ADPKD criteria are not met, multiple simple cysts are most likely responsible for the hepatic cystic lesions.

ADPKD is characterised by an increased risk of developing vascular manifestations. Hypertension occurs in approximately 50%-70% of patients, and almost half of these hypertensive patients are reported to have left ventricular hypertrophy (LVH)[112]. Mitral valve prolapse is observed in 25% of patients and intracranial aneurysms in 4%-12% of patients[112]. As a result, magnetic resonance angiography (MRA) must be performed when ADPKD patients have a positive family history of intracranial aneurysms because the rupture of aneurysms is reported to be responsible for 4%-7% of deaths in affected ADPKD families[113]. In contrast to ADPKD, several studies have shown that PCLD patients do not appear to have an increased risk of vascular malformations. One study involving 19 PCLD cases reported hypertension in 10.5% of cases, mitral valve prolapse in 0% and aneurysms in 5.3%[79]. Another study involving 38 PCLD cases found mitral valve prolapse in 1 case (2.6%)[114]. Subsequently, targeted screening is not advised for PCLD.

Therapy

The main objective of therapy is to reduce liver cyst volume to diminish mass effect-related symptoms[115]. Hence, the only indication for reducing cyst volume is when a PLD patient reports symptoms that can be linked to the polycystic liver[116].

Surgical procedures, such as aspiration-sclerotherapy and fenestration, are indicated when PLD consists of large cysts confined to a limited part of the liver. In more extensive disease, segmental hepatic resection or even liver transplantation is imperative to relieve symptoms[117]. Future medical therapies include somatostatin analogues, as several clinical trials with lanreotide and octreotide achieved polycystic liver volume reduction in PCLD and ADPKD[118-123].

CONCLUSION

Cystic lesions of the liver encompass a wide spectrum of disorders. As a result of the frequent use of abdominal imaging techniques in recent years, the incidence of so-called coincidental cysts has increased. Simple cysts are the most prevalent and have a tendency to follow a benign course. However, complicated cysts, echinococcosis and cystic neoplasms (*e.g.*, cystadenoma and cystadenocarcinoma), which cause a diagnostic enigma, demand accurate diagnosis in the early stage because specific treatment could be required. Furthermore, the presence of multiple hepatic cystic lesions must raise the suspicion of PCLD or ADPKD and requires further screening.

USG remains the most accurate, non-invasive and cost-effective imaging modality for diagnosing simple cysts. Despite recent advances (*e.g.*, contrast-enhanced CT and DWI), distinguishing complicated cysts from echinococcosis and cystic neoplasms remains impossible with USG, CT or MRI alone. Because of an ever-increasing spread of *Echinococcus* to previously non-endemic regions and its initial quiescent phase after primary infection, it is necessary to exclude echinococcosis. Serodiagnostic tests have high sensitivity and specificity to reveal *Echinococcus* antibodies. Subsequently, CEUS can be used to accurately and reliably exclude cystic neoplasms by demonstrating the absence of any enhancement within the hepatic cystic lesion. Therefore, when CEUS is available, it reduces the need for surgical resection.

The detection of multiple liver cysts requires USG screening of both kidneys and extensive family history taking regarding the occurrence of ADPKD or PCLD. When PCLD or ADPKD criteria are not met, multiple simple cysts are most likely responsible for the hepatic cystic lesions. PCLD or ADPKD could eventually be diagnosed through USG follow-up.

To summarise, we developed a diagnostic algorithm by integrating recent advances with conventional diagnostic tools (Figure 4). Our diagnostic algorithm facilitates evidence-based clinical decision making when clinicians are confronted with coincidental hepatic cystic lesions on USG. Further development of USG- and MRI-based techniques, such as CEUS and DWI, will probably lead to further improvement of hepatic cystic lesion characterisation.

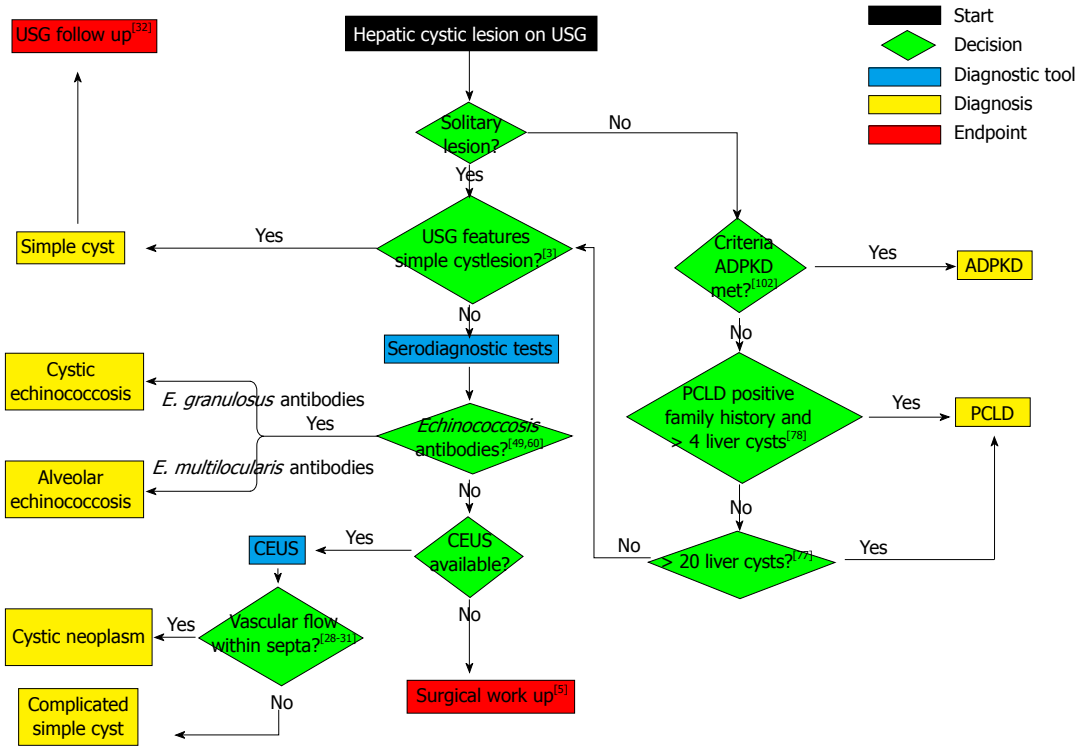

Figure 4 Diagnostic algorithm. Diagnosis of hepatic cystic lesions after detection on ultrasonography. *E. granulosus*: *Echinococcus granulosus*; *E. multilocularis*: *Echinococcus multilocularis*; CEUS: Contrast-enhanced ultrasound; PCLD: Polycystic liver disease; ADPKD: Autosomal dominant polycystic kidney disease.

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MINIREVIEWS

Heme oxygenase-1 and gut ischemia/reperfusion injury: A short review

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Abstract

Ischemia/reperfusion (I/R) injury of the gut is a significant problem in a variety of clinical settings and is associated with a high morbidity and mortality. Although the mechanisms involved in the pathogenesis of gut I/R injury have not been fully elucidated, it is generally believed that oxidative stress with subsequent inflammatory injury plays an important role. Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme, followed by production of CO, biliverdin, and free iron. The HO system is believed to confer cytoprotection by inhibiting inflammation, oxidation, and apoptosis, and maintaining microcirculation. HO-1, an inducible form of HO, serves a vital metabolic function as the rate-limiting step in the heme degradation pathway, and affords protection in models of intestinal I/R injury. HO-1 system is an important player in intestinal I/R injury condition, and may offer new targets for the management of this condition.

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Key words: Heme oxygenase; Ischemia/reperfusion injury; Oxidative stress; Cytoprotection; Gut

Core tip: In this review, we focused on the heme oxygenase (HO)-1 system and its possible roles and mechanisms in gut ischemia/reperfusion (I/R) injury studied to date. This review, for the first time, reviews in detail the relationship between HO-1 and gut I/R injury.

Liao YF, Zhu W, Li DP, Zhu X. Heme oxygenase-1 and gut ischemia/reperfusion injury: A short review. *World J Gastroenterol* 2013; 19(23): 3555-3561 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3555.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3555

INTRODUCTION

Ischemia/reperfusion (I/R) injury of the gut occurs frequently in a variety of clinical settings, including abdominal aortic aneurysm surgery, mesenteric artery occlusion, small bowel transplantation, cardiopulmonary bypass, strangulated hernias, trauma, and shock[1]; the exact mechanisms involved in the pathogenesis of which have not been fully elucidated. Gut I/R injury is associated with a substantial morbidity and mortality[2].

Heme oxygenase (HO) is the rate-limiting enzyme in heme degradation, resulting in the formation of CO, biliverdin, and free iron[3]. There are three distinct HO isoforms (HO-1, HO-2, and HO-3) identified to date. HO-1 is the inducible form of the enzyme, and is expressed in relatively low amounts in most tissues[3]. HO-2 is the constitutive isoform, expressed mainly in brain and testis. HO-3 is identified only in rats, and its physiological

role remains unclear[4].

HO-1, as an inducible form, also belongs to a member of the heat shock protein family and is highly inducible by a vast array of stimuli[3]. Many studies indicated that the induction of HO-1 plays a significant protective role against inflammatory processes and oxidative tissue injury[5-7]. In this review, we focus on the current understanding of the cytoprotective effects observed with the HO system during gut I/R injury. The implications for possible therapeutic manipulation of HO in gut I/R injury are elucidated.

ISCHEMIA/REPERFUSION INJURY IN GUT

Interruption of blood supply results in ischemic injury which rapidly damages metabolically active tissues. Paradoxically, reintroduction of blood flow obtained following ischemia initiates a cascade of events that can potentially worsen the original injury. This effect is known as reperfusion injury[8]. The intestine is composed of labile cells that are very susceptible to I/R injury[9]. Multiple factors have been shown to be involved in the process of intestinal I/R injury. The primary pathophysiological events of this injury involve microcirculatory flow disturbances caused by the production of reactive oxygen species (ROS). Tissue ischemia and oxidative stress activate families of protein kinases that converge on specific transcriptional factors that regulate the expression of inflammatory genes. The resulting gene products include enzymes [*e.g.*, inducible nitric oxide synthase (iNOS); phospholipase A2, and cyclooxygenase-2 (COX-2)], cytokines [*e.g.*, tumor necrosis factor α (TNF-α); interleukin-1 (IL-1); interleukin-6 (IL-6)], prostaglandins (*e.g.*, PGE-2), and adhesion molecules [*e.g.*, intracellular adhesion molecule (ICAM-1); E-selectin][10-14]. These initiate local inflammation, which is further amplified by the recruitment of circulating leukocytes[12], which appear to be key effector cells in causing tissue injury. Furthermore, I/R injury induces widespread endothelial cell apoptosis and the loss of endothelial cells in the vessels serving the organ results in thrombosis[15,16] directly in the intestine[16]. This injury observed during I/R is believed to trigger a systemic inflammatory response leading to multiple organ failure[17,18], which frequently involves the lungs[19,20] and liver[21]. Intestinal I/R injury is a complex, multifactorial pathophysiological process, dependent upon an understanding of which the optimal therapeutic approach is aimed at ameliorating I/R injury (Table 1). HO-1 system might be one of the most promising approaches among the potential therapeutic options.

ROLE OF HO-1 IN ISCHEMIA/REPERFUSION INJURY IN GUT

HO-1 is expressed constitutively in normal gastric, intestinal, and colonic mucosa[22,23], and is up-regulated in their inflamed tissue[23]. Many studies showed that HO-1 is involved in a variety of regulatory and protective cellular mechanisms as a stress-responsive protein[5,6]. The normal expression and up-regulation of HO-1 suggest that activation of HO-1 could act as a natural defensive mechanism to alleviate inflammation and tissue injury in the gastrointestinal tract[24,25]. HO has been shown to have potent cytoprotective effects on intestinal I/R injury as well[26]. For example, induction of HO-1 by cobalt-protoporphyrin administration before intestinal I/R resulted in a significant reduction of intestinal tissue injury[27]. Another enhancer (pyrrolidine dithiocarbamate) of HO production improves intestinal microvascular perfusion and attenuates I/R injury of the intestine, possibly *via* HO production[28]. Similarly, administration with a HO inducer (hemin) results in lessened mucosal injury and improved intestinal transit following gut I/R[29]. Glutamine protects the intestine from warm ischemic injury, which was considered to be associated with inducible HO-1 expression through the interaction with cellular antioxidative activity and the inhibition of cytokines[30]. Several studies demonstrated that intraischemic hypothermia, hypertonic saline resuscitation, and whole-body hyperthermia decrease inflammation and protect against intestinal injury in a model of gut I/R[31-33]. Administration of IL-2, an immunoregulatory cytokine, resulted in clinical improvement of the study animals after intestinal I/R[34]. These protective interventions were associated with the induction of HO-1. Postischemic leukocyte-endothelial cell adhesive interactions are prevented by 5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside preconditioning 24 h prior to I/R in the small intestine by HO-dependent mechanisms[35]. Furthermore, ischemic preconditioning of the intestine might prove to be an effective strategy for the amelioration of I/R injury, in which HO is involved[18,36,37]. Pretreatment with Radix Paeoniae Rubra[38], or the anticancer drug doxorubicin[20], can attenuate acute lung injury resulting from intestinal I/R. These results demonstrate that HO-1 is implicated in cytoprotection and may be an effective agent for the treatment of gut I/R.

MECHANISMS OF ACTION

There is increasing evidence that HO-1 plays an important protective role in gut I/R injury. There are four factors that could be responsible for the protection of HO-1 in intestinal I/R, including: (1) removal of free heme; (2) CO; (3) biliverdin/bilirubin; and (4) Fe^{2+}.

REMOVAL OF FREE HEME

Heme, an essential iron chelate, is a potentially damaging species that not only provides a lipophilic form of iron, but also can directly attack and impair a multiplicity of intracellular targets[39]; production increases under conditions of oxidant stress, especially in I/R injury[39,40]. HO-1 is the key enzyme in heme degradation and plays a key role in regulating the intracellular heme level. HO-1 activity leads to rapid removal of free heme. Thus, in order

Table 1 The role of heme oxygenase-1 for the protection of intestinal ischemia/reperfusion injury

	Treatment	I/R model	Ref.
HO-1			
	Glutamine	Warm ischemia	[30]
	Ischemic preconditioning	Resuscitation after shock	[37]
	Doxorubicin	Warm ischemia	[20]
	Hypothermia	Warm ischemia	[32]
	IL-2	Warm ischemia	[34]
	Hemin	Warm ischemia	[29]
	Hypertonic saline	Warm ischemia	[31]
	Pyrrolidine dithiocarbamate	Warm ischemia	[28]
	Hyperthermia	Warm ischemia	[33]
	Ischemic preconditioning	Warm ischemia	[36]
	Cobalt-protoporphyrin	Warm ischemia	[27]
	Ischemic preconditioning	Endotoxic shock	[18]
	Radix Paeoniae Rubra	Warm ischemia	[38]
	AICAR preconditioning	Warm ischemia	[35]
CO			
	Gas inhalation	Intestinal transplants	[11]
	Gas inhalation	Intestinal transplants	[13]
	Gas inhalation	Intestinal transplants	[14]
	Gas inhalation	Intestinal transplants	[47]
	CO solution	Intestinal transplants	[49]
	CORM preconditioning	Warm ischemia	[48]
	CORM preconditioning	Warm ischemia	[12]
Biliverdin/bilirubin			
	Bilirubin	Warm ischemia	[60]
	Bilirubin	Warm ischemia	[61]
	Biliverdin	Intestinal transplants	[47]

AICAR: 5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside; CORM: CO-releasing molecules; IL-2: Interleukin-2; I/R: Ischemia/reperfusion; HO: Heme oxygenase.

to prevent heme from both extracellular and intracellular sources reacting and producing ROS, the heme degradation step is an important consideration in the cytoprotection afforded by the HO system[40].

CO

CO is one of the three products of heme degradation by HO-1 and has profound effects as a signaling molecule that culminates in anti-inflammatory, antiapoptotic, and vasodilating effects[41,42]. A number of studies have revealed that CO mediates potent cytoprotective and anti-inflammatory effects in models of I/R injury of the heart, lung, kidney, and liver[43-46]. Some studies demonstrate that the efficacy of CO gas inhalation for the prevention of cold intestinal I/R injury using a small intestinal transplantation model, in which CO is able to effectively inhibit an early up-regulation of proinflammatory mediators such as IL-6, IL-1, TNF-α, ICAM-1, iNOS, and COX-2[11,13,14,47]. It has been reported that pre-treatment with CO-releasing molecules also markedly reduced intestinal inflammation induced by surgical manipulation of the small intestine[48] or by occluding the superior mesenteric artery[12]. Similarly, one study showed that cold storage in a preservation solution that was bubbled with 5% CO significantly reduced I/R injury associated with intestinal transplantation, which reduced inflammatory mediator up-regulation and improved graft blood flow[49]. Moreover, CO-treated animals showed early up-regulation of the anti-apoptotic molecule Bcl-2, and down-regulation of the proapoptotic signal Bax, and reduced *in vivo* apoptosis of both vascular endothelial cells and intestinal epithelial cells[11]. The protective effects of CO are arbitrated by activating one or both of the two key signaling pathways. One of the pathways is soluble guanylate cyclase/cyclic guanosine monophosphate and the other one is the p38 mitogen-activated protein kinase pathway which transduces oxidative stress and inflammatory signaling[11,48-53], through which CO exerts significant cytoprotection due to its anti-inflammatory, vasodilating, and anti-apoptotic properties in gut I/R injury.

BILIVERDIN/BILIRUBIN

HO degrades heme into equimolar quantity of biliverdin. Biliverdin is, in turn, very rapidly converted to bilirubin by the enzyme biliverdin reductase[3]. Biliverdin and its reduced product, bilirubin, scavenge various ROS and are hence considered potent antioxidants[54,55], which have been shown to confer cytoprotection against oxidative stress conditions in various cell types[56]. Several studies have also demonstrated that the administration of biliverdin and/or bilirubin is potently cytoprotective in I/R injury of the liver and heart, and in organ transplantation[57-59]. Evidence from an experimental small intestinal I/R injury model in rats describes a protective effect for bilirubin[60], in which the bilirubin is infused *via* the jugular vein. Similarly, another study showed that increased serum bilirubin ameliorates the extent of intestinal IR injury[61]. Recent studies have suggested that biliverdin, in addition to its antioxidant properties, may have anti-inflammatory action. For example, treatment with biliverdin can significantly decrease mRNA expression of iNOS, COX-2, and ICAM-1, as well as the inflammatory cytokines IL-6 and IL-1, and decreased neutrophil infiltration into the jejunal muscularis in rat syngeneic small intestinal transplants[47]. These results suggest that bilirubin possesses complex immune-modulatory and antioxidant effects.

Fe^{2+}

Though HO activity is generally associated with cellular protection, Fe^{2+}, the third product of heme decomposition, participates in the Fenton reaction to promote the generation of ROS and is believed to have potential deleterious effects. Increased iron levels, on the one hand, can upregulate an iron-transporter pump that removes intracellular Fe^{2+} from the cell[62]. On the other hand, iron release from HO activity induces the expression of ferritin (an iron storing protein)[63,64]. Expression of ferritin was originally reported to protect endothelial cells against oxidant damage *in vitro*[64]. In addition, over-expression of H-ferritin (heavy chain ferritin) has also been shown to protect cultured endothelial cells from undergoing apop-

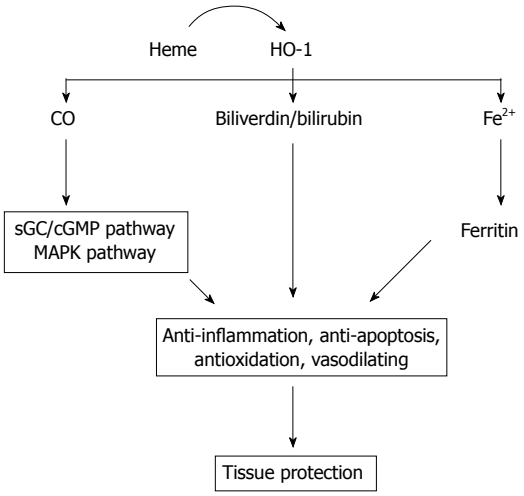

Figure 1 Schematic demonstration of the heme oxygenase-1 system and its biologic activities in gut ischemia/reperfusion injury. HO: Heme oxygenase.

tosis and protects the liver from transplant-associated I/R injury[65]. Thus, ferritin seems to confer cytoprotection against oxidative challenge. There is no information about the roles of iron and ferritin in gut I/R injury, but in such a mechanism they could still be operative.

CLINICAL EVIDENCE

As we mentioned above, the HO-1 system plays an important role in the cytoprotective process, up-regulation of which seems to be a potential therapeutic option for gut I/R injury. As far as we know, there have been no definitive trials designed to evaluate the efficacy of chemical HO-1 inducers in the clinical setting. Hemin, as an inducer of HO-1, has been used extensively in experimental studies, but has only been used by physicians experienced in the management of porphyrias clinically. However, there are increasing reports showing that hemin-induced HO-1 activity is a host defense mechanism in different animal models, such as the thrombosis vascular model[66], in liver I/R injury[67], acute pancreatitis with multi-organ failure[68,69], human immunodeficiency virus-1 infection[70], and spontaneously hypertension[71]. Such disease states share part or common physiopathological process with gut I/R injury, which suggests that hemin could offer a therapeutic benefit for gut I/R injury. A richer understanding of the cytoprotective mechanisms of hemin therapy will be necessary, which will also pave the way for clinical application in the treatment of gut I/R injury.

CONCLUSION

Intestinal I/R injury is a complex, multifactorial pathophysiological process. Despite its complexity, the HO-1 system, owing to its antioxidative, anti-inflammatory, anti-apoptosis, and potent cytoprotective properties (Figure 1), may serve as promising potential therapeutic options for intestinal me/R injury.

The modulation of HO-1 expression using genetic or pharmacological strategies may offer therapeutic strategies for intestinal I/R injury. Furthermore, HO-1-related molecules, including CO and biliverdin/bilirubin, might be employed as drugs in the management of intestinal I/R injury. More importantly, regulating the HO-1 system with different agents has already been demonstrated as important for attenuating I/R injury in other organs including the brain, liver, and kidney[40,72-74]. It is reasonable to assume that such a mechanism could also be operative in intestinal I/R injury. Research focused on the underlying mechanisms for the observed effects of HO-1 and its products will be necessary before their use can be evaluated in clinical applications for the prevention and/or treatment of human diseases such as intestinal I/R injury.

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ORIGINAL ARTICLE

Virulence factors of *Enterococcus* strains isolated from patients with inflammatory bowel disease

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Author contributions: Golińska E performed the majority of the experiments, including the detection of gelatinase activity, measurement of hydrogen peroxide production and the determination of hydrogen peroxide decomposition, and wrote the manuscript; Tomusiak A collected and analysed the data; Gosiewski T performed PCR and multiplex PCR; Więcek G evaluated the adherence to human gut epithelium cells; Machul A and Mikołajczyk D evaluated the biofilm production; Heczko PB and Bulanda M supervised the experiments; and Strus M designed the experiments and supervised the project.

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Abstract

AIM: To determine the features of *Enterococcus* that contribute to the development and maintenance of the inflammatory process in patients with inflammatory bowel disease (IBD).

METHODS: Multiplex polymerase chain reaction (PCR) was applied to assess the presence of genes that encode virulence factors [surface aggregating protein (*asa1*), gelatinase (*gelE*), cytolysin (*cylA*), extracellular surface protein (*esp*) and hyaluronidase (*hyl*)] in the genomic DNA of 28 strains of *Enterococcus* isolated from the intestinal tissues of children with IBD (n = 16) and of children without IBD (controls; n = 12). Additionally, strains with confirmed presence of the *gelE* gene were tested by PCR for the presence of quorum sensing genes (*fsrA*, *fsrB*, *fsrC*) that control the gelatinase production. Gelatinase activity was tested on agar plates containing 1.6% gelatin. We also analysed the ability of *Enterococcus* strains to release and decompose hydrogen peroxide (using Analytical Merckoquant peroxide test strips) and tested their ability to adhere to Caco-2 human gut epithelium cells and form biofilms *in vitro*.

RESULTS: A comparison of the genomes of *Enterococcus* strains isolated from the inflamed mucosa of patients with IBD with those of the control group showed statistically significant differences in the frequency of the *asa1* gene and the *gelE* gene. Furthermore, the cumulative occurrence of different virulence genes in the genome of a single strain of *Enterococcus* isolated from the IBD patient group is greater than in a strain from the control group, although no significant difference was found. Statistically significant differences in the decomposition of hydrogen peroxide and adherence to the Caco-2 epithelial cell line between the strains from the patient group and control group were demonstrated. The results also showed that profuse biofilm production was more frequent among *Enterococcus* strains isolated from children with IBD than in control strains.

CONCLUSION: *Enterococcus* strains that adhere strongly to the intestinal epithelium, form biofilms and possess antioxidant defence mechanisms seem to have the greatest influence on the inflammatory process.

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Key words: *Enterococcus*; Virulence factors; Inflammatory bowel disease; Hydrogen peroxide; Biofilm

Core tip: In this research we have attempted to show

which features make *Enterococcus* strains contributing to the development and maintenance of the inflammatory process in patients with inflammatory bowel disease. The outcome of this research may have an impact on better understanding of the pathomechanisms of this disease, as its etiology is not fully known. The study results suggest that *Enterococcus* strains which adhere strongly to the intestinal epithelium, form biofilm as well as possess the enzymatic mechanisms protecting them against the effects of reactive oxygen species, seem to have the highest chances to survive and influence the inflammatory process.

Golińska E, Tomusiak A, Gosiewski T, Więcek G, Machul A, Mikołajczyk D, Bulanda M, Heczko PB, Strus M. Virulence factors of *Enterococcus* strains isolated from patients with inflammatory bowel disease. *World J Gastroenterol* 2013; 19(23): 3562-3572 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3562.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3562

INTRODUCTION

Inflammatory bowel disease (IBD) refers to two disease entities: ulcerative colitis (UC) and Crohn's disease (CD). UC is a chronic inflammatory process of the mucous membranes of the rectum and colon. Inflammatory changes in the form of recurrent surface ulcerations are located along the whole length of the large intestine. The course of UC course ranges from mild disease activity, lasting for years, to severe disease that can result in death after only a few weeks. CD is a chronic inflammatory process that can affect any part of the alimentary system, from the oral cavity to the rectum.

The aetiology of IBD is not yet fully understood[1]. Based on numerous clinical observations and results of extensive *in vitro* and *in vivo* experiments, it is currently thought that this disease develops as a result of the concurrence of three factors: genetic predisposition, disorders of the immune system and the influence of the environmental factors. Among the environmental factors, changes in the composition of the bacterial flora that colonise the human alimentary tract are considered to be of great influence[2]. Consequently, a great deal of attention is currently devoted to the study of the bacterial species that constitute the normal alimentary tract flora compared to those found in the tract of the IBD patients.

From 2004 to 2006, research on the qualitative and quantitative changes in the microflora of the alimentary tracts of a group of children who had been diagnosed with CD or UC for the first time was performed in our department[3,4]. No single aetiological factor that could be responsible for the exacerbation of the progressing inflammatory process was confirmed based on the obtained results.

However, the analysis of the quantitative changes in the group of children with CD identified a significant increase in the population of cocci (including *Streptococcus* and *Enterococcus)* and bacteria belonging to the genus *Lactobacillus* in the inflammatorily changed sites with a simultaneous decrease in the number of strictly anaerobic bacteria, particularly those belonging to the genus *Bifidobacterium*.

All of the above-mentioned bacteria are important members of the commensal flora of the human colon. Therefore, we must consider the mechanisms by which these bacteria may contribute to the development and/or maintenance of the inflammatory process in patients suffering from IBD. The virulence potential of the genus *Enterococcus* has been established in many publications, and it is this genus contributes to infections such as peritonitis, bloodstream and urinary tract infections and endocarditis[5,6]. Their potential role in the pathomechanisms leading to IBD has also been highlighted by research utilising *IL-10* gene knockout mice to show that *Enterococcus faecalis* (*E. faecalis)* can induce IBD[7].

Recent studies on the pathogenicity of enterococci indicate that the genomes of strains that are able to cause tissue damage and inflammation contain a pathogenicity island that encodes aggregation substance (AS), gelatinase, extracellular surface proteins (Esp), cytolysin, hyaluronidase and other proteins[8,9]. Enterococci that express AS were found to resist phagocytosis significantly better than an isogenic AS-negative strain by inhibiting the respiratory burst of macrophages[10]. Gelatinase, a protease produced by enterococci, is capable of hydrolysing gelatin, collagen, casein, haemoglobin and other peptides[9]. The Esp enhance biofilm formation in *E. faecalis*[11]. Cytolysin produced by the enterococci is lethal for a broad range of prokaryotic and eukaryotic cells[12]. Hyaluronidase is mainly a degradative enzyme that is associated with tissue damage[13].

Little attention has been devoted to the ability of enterococci to release hydrogen peroxide into the extracellular space[14]. Pursuant to the results obtained previously by our group, select members of several genera, including *Streptococcus, Enterococcus* and *Lactobacillus*, are, under aerobic conditions, able to produce amounts of hydrogen peroxide comparable to those released by cells of the immune system during the oxidative burst[15]. This additional source of hydrogen peroxide could help sustain, or even exacerbate, gut inflammation[16]. Notably, certain *Enterococcus* strains can defend themselves against the surplus of reactive oxygen species (ROS) by producing antioxidative enzymes to increase their chances of survival in unfavourable conditions[17].

Thus, the main objective of this work was to compare the occurrence of genes encoding selected virulence factors [surface aggregating protein (*asa1*), gelatinase (*gelE*), cytolysin (*cylA*), extracellular surface protein (*esp*) and hyaluronidase (*hyl*)] in the genomes of *Enterococcus* strains isolated from patients suffering from IBD with the occurrence of these genes in the genomes of strains derived from the control group subjects. Additionally, strains confirmed positive for the *gelE* gene were tested for the presence of the quorum sensing genes *fsrA-C*

that regulate gelatinase production, and gelatinase activity was tested on agar plates containing 1.6% gelatin. The adherence of the *Enterococcus* strains to Caco-2 epithelial cells and their ability to form biofilms were also tested. Furthermore, the ability of strains isolated from inflamed or non-inflamed gut mucosa to release and decompose extracellular hydrogen peroxide was assessed.

MATERIALS AND METHODS

Bacterial strains

Enterococcus strains were isolated from colon biopsies of 34 children who were diagnosed with IBD for the first time and 24 patients from the control group comprised of children with functional bowel disorders that were hospitalised in the same hospital during the same period of time. Neither the IBD patients nor the participants in the control group received antibiotics during the two weeks prior to the study. The diagnosis of CD or UC was based on endoscopic, histopathological, immunological and radiological criteria[3,4]. The biopsies were taken from both groups of patients during colonoscopy procedures carried out at the Clinic of Paediatrics, Gastroenterology and Nutrition of the Polish-American Institute of Paediatrics of the Jagiellonian University Medical College in Cracow pursuant to the approval of the Bioethical Committee (No. KBET/236/B/2002). The patients were prepared for the colonoscopy using routine washing procedures described by Gosiewski *et al*[4]. During the colonoscopy, the location and intensity of the lesions was assessed, and tissue fragments were obtained for histopathological and microbiological examinations. The biopsy samples were transported to the microbiological laboratory in Schaedler broth (SAB, Difco, United States, + 10% glycerol) at -20 ℃.

In the laboratory, the frozen samples were thawed, weighed and homogenised in 1 mL of SAB and quantitatively analysed for the main cultivable bacterial constituents of the colon microflora using differential media in aerobic and anaerobic conditions[3,4]. These steps were performed aseptically in an anaerobic chamber (MACS - MG 500 Work Station, DW Scientific, Shipley, United Kingdom) in N_2 (85%) + H_2 (10%) + CO_2 (5%) atmosphere. The homogenised samples were serially diluted with SAB and 100 μL aliquots plated on the following media: McConkey Agar (Oxoid, Basingstoke, United Kingdom) for *Enterobacteriaceae*, Columbia Blood Agar (Difco, Lawrence, Kansas, United States) with 5% sheep blood for streptococci, Enterococcosel agar (BBL, BD, Franklin Lakes, United States) for enterococci, MRS agar (Oxoid) for lactobacilli and other lactic acid bacteria, BL agar for bifidobacteria and Wilkins-Chalgren agar base (Difco) with supplements for *Bacteroides*.

The morphology of the grown colonies was analysed using a magnifying glass, and several colonies of each morphological type were subcultured on appropriate aerobic and anaerobic media and subsequently Gram-stained. After further incubation and culture purity checks, the phenotypic identification was conducted with the use of commercial identification systems (API 20E, API20A, APIStaph, APIStrep, bioMerieux, Marcy l'Etoile, France; BBL Crystal ID System, BD, Franklin Lakes, United States). The identification was confirmed by species-specific multiplex polymerase chain reaction (PCR) as described by Jackson *et al*[18].

The selected strains were preserved at -80 ℃ on glass beads in BBL Nutrient Broth with the addition of 15% glycerol.

Multiplex PCR

To detect presence of genes encoding selected virulence factors (*asa1*, *gelE, cylA, esp, hyl*) in the genomes of the tested strains, multiplex PCR was applied according to the methods of Vankerckhoven[19].

PCR

To investigate the presence of quorum sensing genes (*fsrA, fsrB, fsrC*) in the genomes of all the strains positive for the *gelE* gene, PCR was applied pursuant to Qin *et al*[20].

The reference strain *E. faecalis* ATTC29212 containing the gene *fsrC* was used as the positive control. The negative control was a reference strain of *E. faecium* (ATTC 35667) that did not possess any of the examined genes.

The product sizes for *fsrA*, *fsrB* and *fsrC* were 484 bp, 574 bp and 580 bp, respectively (BLAST).

Detection of gelatinase activity

The production of gelatinase in *Enterococcus* strains was detected by the method described by Steck *et al*[6]. Supernatants from overnight cultures were spotted onto tryptic soy agar supplemented with 0.5 g/L *L*-cysteine and 1.6% Difco gelatine. The zone of clearance was measured after 24 h of incubation.

Adherence to human gut epithelium cells and evaluation of biofilm production

The ability of *Enterococcus* strains to adhere to Caco-2 human gut epithelium cells (ATCC HTB-37) was assessed using an *in vitro* assay according to Górska-Frączek[21]. Caco-2 cells were cultured for 24 h in a 12-well flat bottom tissue culture plate at a density of 1×10^6 cells/mL (Iwaki, Japan) in Eagle's 1959 medium (MEM) with *L*-glutamine and $NaHCO_3$ (IITD, Wroclaw, Poland) containing 5% foetal calf serum (Sigma-Aldrich Chemie, Germany) and antibiotics (penicillin 100 UI/mL, streptomycin 100 UI/mL, neomycin 200 μg/mL) (Sigma Aldrich Chemie, Germany) and were washed twice with PBS. Overnight cultures of bacteria were diluted with MRS+MEM to a concentration of about 10^8 CFU/mL. The Caco-2 cells were inoculated with the bacterial cultures. After incubating at 37 ℃ for 30 min, wells were washed twice with PBS to release unbound bacteria. Then, the cells were fixed with 3.7% formaldehyde for 1 h, washed twice with PBS and stained with crystal violet stain (Merck, Germany). The degree of adhesion was evaluated using a semiquantitative scoring system ranging from (-) to (+++). The adherent *Enterococcus* cells were

counted in 20 randomly selected microscopic fields, as we have previously described[22].

Fifteen strains of enterococci collected from children with IBD and 10 strains from the control children were examined for biofilm formation in sterile plastic 96-well plates with an adherent surface (Greiner Bio-One, United States). Bacterial cells were grown in 10 mL of trypticase soy broth (TSB, Difco) at 37 ℃ for 24 h in aerobic conditions. The culture was then centrifuged (2000 r/min; 10 min) and washed with 10 mL of saline. A suspension of each strain (1×10^5 CFU/mL) was prepared by serial dilution of bacteria in saline using MacFarland's scale, and then 20 μL of the prepared suspension was added to a well followed by 180 μL of sterile TSB. The final concentration of bacteria was 1×10^4 CFU/mL. The plates were centrifuged for 10 min at 2000 r/min to sediment the bacteria onto the bottom of each well and were then incubated for 48 h (37 ℃, aerobic conditions). The biofilm quantity (total mass of bacterial polysaccharides) was measured using Congo red dye according to a modified procedure described by Reuter *et al*[23]. Briefly, at two different time points (0 and 48 h), the plates were centrifuged, the culture medium was gently removed from the wells and 200 μL of 0.1% Congo red solution was immediately added. The plates were left at room temperature for 30 min and then washed twice with buffered saline to remove unbound dye. The absorbance was measured at a wavelength of 492 nm using a spectrophotometer (Awareness Technology, Inc.). All measurements were performed in triplicate, and the mean values ± SD were calculated. The degree of biofilm production by the tested cocci was arbitrarily categorised as either highly positive ($A_{492} \geqslant 0.81$) or weakly positive ($A_{492} \leqslant 0.8$).

Measurement of hydrogen peroxide production

Analytical Merckoquant peroxide test strips (Merck, NJ, United States) were used to measure H_2O_2 production by the tested strains on a detection scale between 0 and 100 mg/L. The tested bacteria were suspended in 2 mL of TSB broth (TSB) (Difco, Kansas, United States) and cultured at 37 ℃ in aerobic conditions. The measurements of H_2O_2 were performed after 4 and 24 h according to the procedure provided by the manufacturer. The mean density of bacterial growth at 4 h was approximately 3×10^6 CFU/mL and increased to 1×10^7 CFU/mL after 24 h. Uninoculated TSB broth was used as a negative control. The amounts of H_2O_2 are presented as mmol/L.

Kinetics of the decomposition of hydrogen peroxide

The bacterial strains were cultured in 10 mL of TSB for 24 h at 37 ℃ in aerobic conditions. Their final density was approximately 0.5 on the McFarland scale. After 24 h, chemically pure hydrogen peroxide (Sigma-Aldrich, United States) was added to each test culture at a final concentration of 2 mmol/L. The culture was incubated under the same conditions for 4 h, and the amount of hydrogen peroxide remaining in the test tube was then determined using Analytical Merckoquant peroxide test strips (Merck, NJ, United States). The resulting amount of H_2O_2 was converted from mg/L into mmol/L. Then, the number of bacterial cells in each culture was determined using the viable count method. These levels were comparable for all strains and equalled approximately 8×10^6 CFU/mL. The negative control was sterile TSB containing 2 mmol/L H_2O_2.

Statistical analysis

The statistical analysis was focused on comparison of probabilities of the analyzed genes presence between the group of the bacteria isolated from IBD children and this from control group. Such comparison was done using frequency χ^2 test. If the given frequencies didn't fulfill the assumption of the test it's less strong equivalents: likelihood ratio of Fisher's exact test were used. The value $P < 0.05$ was regarded as the threshold for statistical significance. All calculations were performed using JMP 7.0.2 (SAS, United States) software package.

RESULTS

In total, 48 strains belonging to the genus *Enterococcus* were isolated. Of these, 34 strains originated from children diagnosed with IBD, including 21 from children diagnosed with CD and 13 from children with UC. In this group, 16 (47%) strains belonged to *E. faecalis*, 10 (29%) to *E. avium* and 8 (24%) to *E. faecium*. In the control group, 5 (36%) out of 14 strains were identified as *E. faecalis*, 4 (29%) as *E. avium*, 3 (21%) as *E. faecium* and 2 (14%) as *E. durans* (Table 1).

For further studies, 28 *Enterococcus* strains were randomly selected, including 16 strains from patients with IBD (n = 9 CD, n = 7 UC) and 12 strains from the control group (Tables 2, 3 and 4). Molecular typing of all *Enterococcus* isolates using PFGE procedure (not described here) was performed before selection to eliminate redundant pulsotypes. No clustering within individual species or across the entire set of strains was noted.

While identifying genes coding for selected virulence factors (*asa1*, *gelE*, *cylA*, *esp*, *hyl*) in the genomes of the *Enterococcus* strains isolated from the inflamed mucosa of IBD patients and from control group patients, statistically significant differences in the frequencies of the *asa1* gene, which encodes the surface aggregating proteins, and the *gelE* gene (P = 0.0091), which encodes the gelatinase that is responsible for decomposing collagen and elastin, were confirmed (Tables 2 and 3; Figures 1 and 2).

The *cylA* gene, which encodes cytolysin, was detected in 2 strains of *E. faecalis* and 1 strain of *Enterococcus faecium* isolated from the inflamed sites. This gene was not detected in the control strains. The obtained results were at the limit of statistical significance (P = 0.0569). The *esp* gene encoding the extracellular surface protein was present in 11 strains isolated from patients with IBD and in 5 strains isolated from the control group. None of the 28 strains examined, including strains from both research groups, contained the *hyl* gene that encodes hy-

Table 1 List of *Enterococcus* strains used in the experiment

Number of isolates	Strain number	Species (API 20 Strep/ Multiplex PCR)	Disease entity/ Patient number
1	3A	*Enterococcus faecium*	CD/1
2	3B	*Enterococcus faecium*	
3	10A	*Enterococcus avium*	CD/2
4	12A/1	*Enterococcus faecalis*	
5	12A/2	*Enterococcus avium*	CD/3
6	12B	*Enterococcus faecium*	
7	25A	*Enterococcus faecium*	CD/4
8	25B	*Enterococcus avium*	
9	31B	*Enterococcus faecalis*	CD/5
10	42A/1	*Enterococcus faecalis*	CD/6
11	42A/2	*Enterococcus faecium*	
12	51A/1	*Enterococcus faecalis*	CD/7
13	51A/2	*Enterococcus avium*	
14	57A	*Enterococcus faecium*	CD/8
15	8A	*Enterococcus faecalis*	CD/9
16	8B/1	*Enterococcus faecalis*	
17	8B/2	*Enterococcus avium*	
18	19B	*Enterococcus faecium*	CD/10
19	22B	*Enterococcus faecalis*	CD/11
20	69A	*Enterococcus faecalis*	CD/12
21	79A	*Enterococcus faecalis*	CD/13
22	32B	*Enterococcus faecalis*	UC/1
23	35A	*Enterococcus faecalis*	UC/2
24	40A	*Enterococcus avium*	UC/3
25	40B	*Enterococcus avium*	
26	48A	*Enterococcus faecalis*	UC/4
27	58A	*Enterococcus faecalis*	UC/5
28	77A	*Enterococcus faecalis*	UC/6
29	33B	*Enterococcus avium*	UC/7
30	29B	*Enterococcus avium*	UC/8
31	34A/1	*Enterococcus faecium*	UC/9
32	34A/2	*Enterococcus avium*	
33	64B	*Enterococcus faecalis*	UC/10
34	65A	*Enterococcus faecalis*	UC/11
35	5B	*Enterococcus faecalis*	Control/1
36	7B	*Enterococcus faecium*	Control/2
37	15B/1	*Enterococcus avium*	Control/3
38	15B/2	*Enterococcus durans*	Control/4
39	16B/1	*Enterococcus avium*	Control/5
40	16B/2	*Enterococcus durans*	
41	27B/1	*Enterococcus faecalis*	Control/6
42	27B/2	*Enterococcus faecium*	
43	27B/3	*Enterococcus avium*	
44	30B/1	*Enterococcus faecium*	Control/7
45	30B/2	*Enterococcus avium*	
46	46B	*Enterococcus faecalis*	Control/8
47	55B	*Enterococcus faecalis*	Control/9
48	66B	*Enterococcus faecalis*	Control/10

CD: Crohn's disease; UC: Ulcerative colitis; PCR: Polymerase chain reaction Species identification was conducted with the use of API Strep system (bioMerieux) and multiplex polymerase chain reaction assays.

aluronidase. No statistically significant differences were observed in the prevalence of *esp* or *hyl* between the two patient groups (Figures 1 and 2).

The cumulative occurrence of different virulence genes in the genome of a single strain of *Enterococcus* isolated from patients suffering from IBD was greater than that of a strain isolated from the control group (mean of 2.6 *vs* 2.0, respectively), but no significant difference was found. For instance, the strains *E. faecalis* 35A and *E. faecium* 57A were isolated from patients with IBD and possessed as many as 4 virulence genes (*cylA, esp, asa1* and *gelE*), but strains containing all four of these genes were not observed in *Enterococcus* isolates from the control group.

Further analysis was performed on strains in which the presence of the *gelE* gene was confirmed (n = 19). Based on the results of a PCR assay for the presence of the regulator genes *fsrA-C,* statistically significant differences between the two groups were noted for the occurrence of *fsrC*. This gene was confirmed in 8 *Enterococcus* strains isolated from the study group, but it was detected in only 2 strains from the control group (P = 0.0195). Furthermore, the presence of *fsrA* was confirmed in 3 strains from the study group and in 1 *Enterococcus* strain from the control group. The *fsrB* gene was detected in the genome of 3 strains isolated from the IBD children and in 2 strains from the control group children. These results are shown in Table 2.

Enterococci containing the gene encoding gelatinase also underwent an *in vitro* test in which the activity of gelatinase on agar plates containing 1.6% gelatin was examined. Zones of clearance were demonstrated only on plates inoculated with cultures of strains that had all three regulator genes (*fsrA, B and C*): 2 *Enterococcus* strains from the studied group and 1 strain from the control group. No statistically significant differences were shown between these two groups. The results are shown in Table 2.

While testing the ability of the enterococci to adhere to Caco-2 cells, statistically significant differences were demonstrated between the bacteria isolated from IBD children compared to controls. Ten of the 24 enterococcal strains from the IBD group strongly adhered to the Caco-2 cells (P = 0.0238). Only 1 strain from the control group adhered to the epithelial cell line used in the study. It is important to note that adherence was demonstrated by all strains having the *asa1* gene, which encodes the surface aggregating protein and allows for increased adherence of the bacteria to the host's tissues. The results of this study are shown in Table 3. However, 6 strains from the control group and 1 strain from the study group with no confirmed presence of *asa1* gene also adhered to the cells. Therefore, there are other virulence factors that increase bacterial adherence to host tissues.

All of the tested *Enterococcus* strains were able to form biofilms structure within 48 h. However, the results demonstrated that profuse biofilm production was more frequent among strains isolated from children with IBD than in control strains. Among the enterococcal strains isolated from the studied group, 11 (73.3%) were abundant biofilm producers (high positive; $A_{492} \geqslant 0.81$), and 4 (26.7%) were weak biofilm producers (low positive; $A_{492} \leqslant 0.8$). Of the 10 strains isolated from the control group, 2 (20%) were classified as abundant biofilm producers and 8 (80%) were weak producers. The difference between the IBD and control groups was not statistically significant. The results of the biofilm formation analysis are shown in Table 3 and Figure 3.

While analysing the ability of the strains to produce

Table 2 Presence of the *gelE* gene, quorum sensing genes (*fsrA*, *fsrB*, *fsrC*) and gelatinase activity in tested *Enterococcus* strains

Source	Strains	Detection of genes				Gelatinase activity on 16% gelatin plates
		gelE	*fsrA*	*fsrB*	*fsrC*	
Crohn's disease	*Enterococcus faecalis 12A/1*	+	-	-	+	-
	Enterococcus faecalis 31B	+	-	+	+	-
	Enterococcus faecalis 51A/1	+	-	-	+	-
	Enterococcus faecium 3A	+	+	+	+	+
	Enterococcus faecium 57A	+	-	-	-	-
	Enterococcus faecium 19B	+	+	-	-	-
	Enterococcus avium 51A/2	-	-	-	-	-
	Enterococcus avium 25B	+	-	-	+	-
	Enterococcus avium 10A	+	-	-	-	-
Ulcerative colitis	*Enterococcus faecalis 35A*	+	-	-	-	-
	Enterococcus faecalis 48A	+	-	-	+	-
	Enterococcus faecalis 77A	+	-	-	+	-
	Enterococcus faecalis 32B	+	+	+	+	+
	Enterococcus avium 40A	+	-	-	-	-
	Enterococcus avium 34A/2	-	-	-	-	-
	Enterococcus avium 40B	+	-	-	-	-
Control	*Enterococcus faecalis 5B*	+	-	-	+	
	Enterococcus faecalis 27B/1	+	+	+	+	+
	Enterococcus faecalis 46B	+	-	+	-	-
	Enterococcus faecalis 55B	-	-	-	-	-
	Enterococcus faecalis 66B	-	-	-	-	-
	Enterococcus faecium 27B/2	+	-	-	-	-
	Enterococcus faecium 30B	-	-	-	-	-
	Enterococcus avium 15B/1	-	-	-	-	-
	Enterococcus avium 16B/1	-	-	-	-	-
	Enterococcus avium 27B/3	+	-	-	-	-
	Enterococcus durans 15B/2	-	-	-	-	-
	Enterococcus durans 16B/2	-	-	-	-	-
ATCC control	*Enterococcus faecalis 29212*	+	-	-	+	-
	Enterococcus faecium 35667	-	-	-	-	-

+: Presence of gene and/or gelatinase activity; -: Lack of gene and/or gelatinase activity Denotes significant (P = 00195 *vs* control group) differences in the occurrence of the *fsrC* gene between the study group and the control group.

extracellular hydrogen peroxide, we observed that only *E. faecium* and *E. avium* species released hydrogen peroxide in amounts equal to or higher than 0.3 mmol/L. In total, 5 of the 16 *Enterococcus* strains isolated from patients with IBD and 3 of the 12 strains isolated from the control group were able to produce extracellular hydrogen peroxide, but this difference was not statistically significant (Table 4).

On the other hand, significant differences in the ability of the strains to decompose H_2O_2 were observed upon examination of the 28 strains of *Enterococcus* isolated from both groups. Two subgroups could be distinguished based on the amount of decomposed hydrogen peroxide. The first group contained enterococci that decomposed 2 mmol/L of hydrogen peroxide to a level of 0-1 mmol/L in 4 h. The second group comprised strains that decomposed hydrogen peroxide to a level higher than 1 mmol/L. The difference in hydrogen peroxide decomposition ability was statistically significant between the *Enterococcus* strains isolated from patients with IBD and the strains isolated from control group patients (P = 0.04; Figure 4).

DISCUSSION

Recently, an important role has been ascribed to the quantitative changes in the composition of the intestinal flora during inflammation[15,24]. These changes are likely related to the alterations in structure and function of the gut epithelium under inflammatory conditions that affect oxygenation, acidity and the functions of many enzymes and may lead to the preferential selection of microbial strains that are able to adapt to and/or interact with the inflamed epithelium.

In this work, *Enterococcus* strains were chosen for further studies because of their potential to be highly virulent. We sought to detect the differences between the virulence potential of the *Enterococcus* strains that colonised the intestines of children with IBD and control strains isolated from children without signs of gut inflammation.

The virulence of *Enterococci* can be linked to the presence of specific virulence factors encoded by specific genes, including cytolytic toxin, extracellular surface protein and AS, serine protease, gelatinase, cell wall adhesins, collagen-binding proteins and capsular polysaccharides[19,25].

In the presented study, statistically significant differences were demonstrated for the occurrence of the *asa1* gene in the population of *Enterococcus* strains isolated from gut biopsies taken from patients with IBD in comparison to those cultured from those of the control

Table 3 Presence of *asa1* gene, degree of adhesion to human gut epithelium cells and biofilm production among *Enterococcus* strains

Source	Strains	*asa1*	Adherence	Biofilm production (after 48 h) $A_{492\ nm}$ ± SD
Crohn's disease	*Enterococcus faecalis 12A/1*	-	+++	0.995 ± 0.351
	Enterococcus faecalis 31B	-	+++	1.302 ± 0.585
	Enterococcus faecalis 51A/1	-	++	1.083 ± 0.413
	Enterococcus faecium 3A	-	-	1.286 ± 0.088
	Enterococcus faecium 57A	+	+++	0.698 ± 0.066
	Enterococcus faecium 19B	+	+++	1.087 ± 0.368
	Enterococcus avium 51A/2	-	-	2.101 ± 0.424
	Enterococcus avium 25B	-	-	2.474 ± 0.683
	Enterococcus avium 10A	-	-	1.423 ± 0.284
Ulcerative colitis	*Enterococcus faecalis 35A*	+	+++	1.528 ± 0.326
	Enterococcus faecalis 48A	-	+++	0.767 ± 0.582
	Enterococcus faecalis 77A	-	++	0.822 ± 0.196
	Enterococcus faecalis 32B	+	++	0.557 ± 0.344
	Enterococcus avium 40A	-	+++	0.859 ± 0.152
	Enterococcus avium 34A/2	-	-	
	Enterococcus avium 40B	-	-	0.282 ± 0.078
Control	*Enterococcus faecalis 5B*	-		0.512 ± 0.065
	Enterococcus faecalis 27B/1	-	-	0.934 ± 0.103
	Enterococcus faecalis 46B	-	++	0.507 ± 0.095
	Enterococcus faecalis 55B	-	-	0.408 ± 0.242
	Enterococcus faecalis 66B	-	-	0.172 ± 0.031
	Enterococcus faecium 27B/2	-		0.917 ± 0.269
	Enterococcus faecium 30B	-	-	0.359 ± 0.087
	Enterococcus avium 15B/1	-	-	
	Enterococcus avium 16B/1	-	-	0.305 ± 0.104
	Enterococcus avium 27B/3	-	-	0.657 ± 0.182
	Enterococcus durans 15B/2	-	-	
	Enterococcus durans 16B/2	-	-	0.315 ± 0.246
ATCC control	*Enterococcus faecalis 29212*	+	+++	0.877 ± 0.221
	Enterococcus faecium 35667	-	++	0.722 ± 0.371

Adhesion degree was assessed as +++ (strong adhesive abilities); ++ (average adhesive abilities) and - (lack of adhesive abilities). Biofilm formation was measured in triplicate at two time points (0 and 48 h). The differences between the time points were averaged, and the mean ± SD are shown.

group without gut inflammation. The *asa1* gene is responsible for bacterial aggregation on the surface of the host tissues (particularly enterocytes, neutrophil granulocytes and epithelial cells of the urinary tract), and it also enables conjugation between bacteria due to increased hydrophobicity of the cell wall surface[26]. Consequently, strains that possess the *asa1* gene can develop large aggregations of bacterial cells during an infection and may simultaneously increase the bacterial population[27]. This property is likely to be important in the pathomechanism underlying IBD because during episodes of diarrhoea, one of the main symptoms of IBD, the majority of the alimentary tract flora is removed with the intestinal contents. Enterococci that express the *asa1* gene are able to strongly adhere to intestinal surfaces lacking a mucous layer and are able to form biofilms for protection against unfavourable environmental factors.

Furthermore, experiments using a rat model of endocarditis have shown that enterococci equipped with the *asa1* gene are better able to survive inside stimulated immune cells, indicating that they may possess enzymatic mechanisms that protect them against the influence of ROS secreted by macrophages during the respiratory burst[10].

The *gelE* gene was more frequently found in the genomes of enterococci isolated from the group of children with IBD. However, the presence of the *gelE* gene alone is not sufficient for the production of gelatinase protein by bacteria. Therefore, we also detected the quorum sensing genes that regulate gelatinase production. Gelatinase is a zinc-dependent metalloproteinase that is capable of hydrolysing gelatin, collagen, casein, haemoglobin and human endothelin[28]. Its activity is one of the main causes of the pathological changes in the host's body upon infection with *E. faecalis*[25]. It is therefore possible that colonisation of the alimentary tract of patients with IBD with *Enterococcus* strains possessing the *gelE* gene can weaken the tight junction protein connections between the epithelial cells lining the intestinal walls of the host, thus leading to the disruption of the mucous barrier[6].

Cytolysin, encoded by the *cylA* gene, contributes to another pathomechanism of IBD that causes the excessive lysis of erythrocytes, leading to the uncontrolled release of large amounts of haemoglobin and subsequently heme and iron ions that influence the populations of *Enterobacteriaceae,* which are able to acquire iron[29]. Therefore, the increased number of enterococci that are able to decompose haemoglobin may be related to increases in the *E. coli* population observed in patients with IBD[30].

Another *Enterococcus* virulence factor examined in this study was the *esp* gene, which encodes the extracellular

Table 4 Mean amounts of hydrogen peroxide (in mmol/L) produced *in vitro* after 4 and 24 h by the tested strains

Source	Strains	H_2O_2 production (mmol/L) after 4 h	H_2O_2 production (mmol/L) after 24 h
Crohn's disease	*Enterococcus faecalis 12A/1*	0	0
	Enterococcus faecalis 31B	0	0
	Enterococcus faecalis 51A/1	0	0
	Enterococcus faecium 3A	0	0
	Enterococcus faecium 57A	0	0
	Enterococcus faecium 19B	0	0.3
	Enterococcus avium 51A/2	0	0.3
	Enterococcus avium 25B	0	0.3
	Enterococcus avium 10A	0.3	1
Ulcerative colitis	*Enterococcus faecalis 35A*	0	0
	Enterococcus faecalis 48A	0	0
	Enterococcus faecalis 77A	0	0
	Enterococcus faecalis 32B	0	0
	Enterococcus avium 40A	0	0
	Enterococcus avium 34A/2	0	0.3
	Enterococcus avium 40B	0	0
Control	*Enterococcus faecalis 5B*	0	0
	Enterococcus faecalis 27B/1	0	0
	Enterococcus faecalis 46B	0	0
	Enterococcus faecalis 55B	0	0
	Enterococcus faecalis 66B	0	0
	Enterococcus faecium 27B/2	0	0.3
	Enterococcus faecium 30B	0	0
	Enterococcus avium 15B/1	0.3	1
	Enterococcus avium 16B/1	0	0
	Enterococcus avium 27B/3	0	0.3
	Enterococcus durans 15B/2	0	0
	Enterococcus durans 16B/2	0	0

The measurement of H_2O_2 was performed with the use of colorimetric method: Merckoquant peroxide test strips.

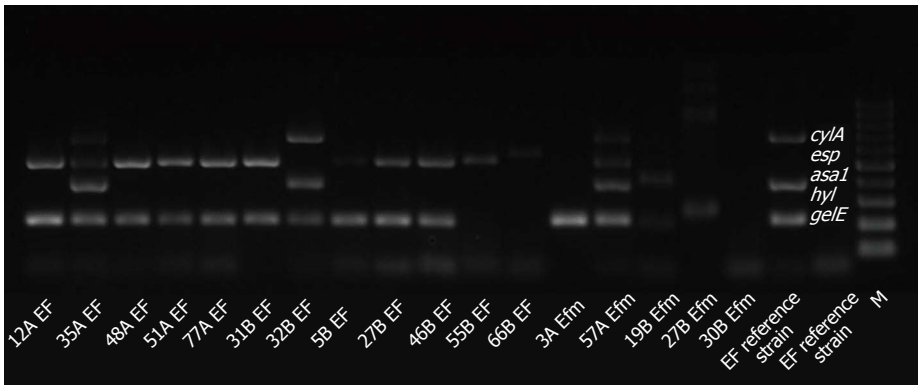

Figure 1 Agarose gel electrophoresis of multiplex polymerase chain reaction amplification products from *Enterococcus* species isolates. The reference strain *Enterococcus faecalis* (*E. faecalis)* ATTC29212 (EF reference strain) possessing the genes *cylA, asa1* and *gelE* constituted the positive control. The negative control was a reference strain of *Enterococcus faecium (E. faecium)* ATTC 35667 Efm reference strain that did not possess any of the examined genes. Product sizes were as follows, *asa1:* 375 bp; *gelE*: 213 bp; *cylA*: 688 bp; *esp*: 510 bp; *hyl*: 276 bp. EF: Tested strains of *E. faecalis*; Efm: Tested strains of *E. faecium;* M: Mass marker 100-1000 bp.

surface protein (Esp). The *esp* gene was present in 11 strains of enterococci from the group of children with the IBD and in 5 strains from the control group. This finding may indicate that enterococci found in biopsy samples from ulcerations were able to adhere to and colonise the intestinal tissues more easily because Esp promotes primary attachment and biofilm formation in *E. faecalis,* as proved demonstrated by Toledo-Arana *et al*[31].

It should be noted here that our studies on the selected virulence factors were based only on the detection of the specified genes that encode these factors, and more information could be obtained from the direct detection of these factors in bacteria using enterocytes in *in vitro* systems or in animal experiments because the expression of the studied genes may be specifically altered in inflamed tissues[32].

In the alimentary tract of patients suffering from IBD, there can be a local accumulation of large amounts of ROS released by stimulated phagocytic cells, such as macrophages and neutrophils, due to the progressing

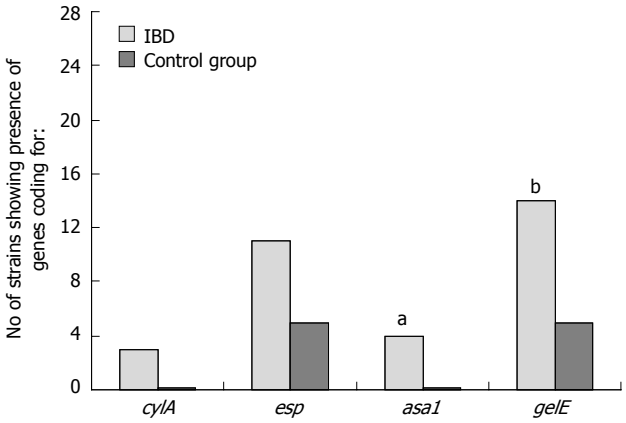

Figure 2 Presence of virulence factors in *Enterococcus* strains. Significant differences in the occurrence of the *asa1* gene ($^aP = 0.0258$ *vs* control group) and the *gelE* gene ($^bP = 0.0091$ *vs* control group) were detected between strains isolated from children with inflammatory bowel disease (IBD) and strains from the participants in the control group.

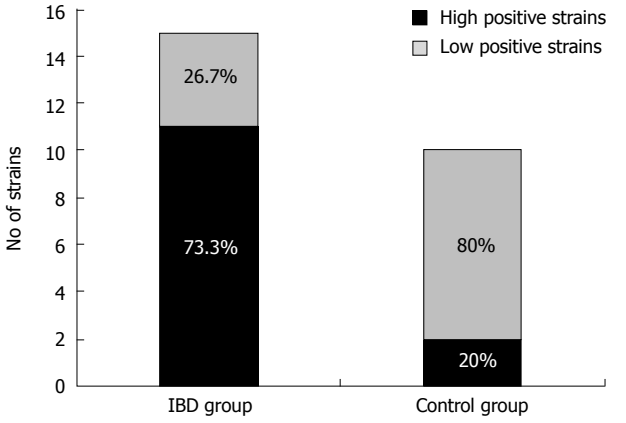

Figure 3 Comparison of the ability of the tested *Enterococcus* strains to form biofilms. The biofilm-forming abilities were assessed after 48 h, and all tested strains were classified as either abundant biofilm producers (high positive strains) or weakly adherent isolates (low positive strains). IBD: Inflammatory bowel disease.

acute and subsequently chronic inflammatory process. Additionally, some species of *Lactobacillus, Streptococcus* and *Enterococcus* that colonise the human intestinal tract are able to produce substantial amounts of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) that are comparable to those released by immune cells[33,34]. However, as has been shown by Al-Mushrif and Jones, the oxygen concentration present in the specific ecological niche regulates the bacterial production of hydrogen peroxide[35]. As we have previously demonstrated and discussed elsewhere (Strus *et al*[15]), the oxygen tension in inflamed tissues during the course of IBD enables the propagation of facultative aerobes such as *Enterococcus* spp. and the production of considerable amounts of hydrogen peroxide and other metabolites. Selected *Enterococcus* strains belonging to the species *E. avium* and *E. faecium* isolated from patients with IBD and children from the control group were able to produce extracellular hydrogen peroxide in our studies. This result may indicate that the production of hydrogen

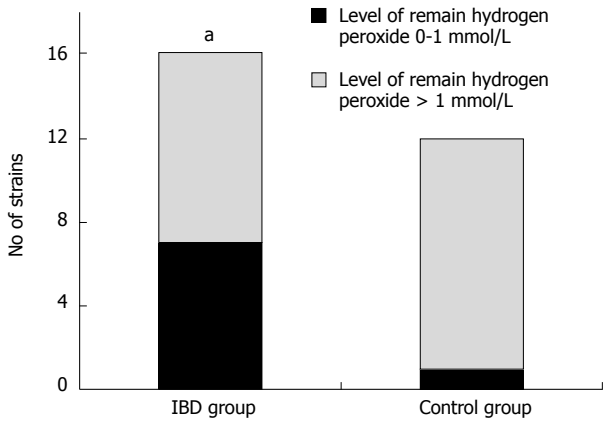

Figure 4 Decomposition of hydrogen peroxide by the tested strains. Statistically significant differences ($^aP = 0.04$ *vs* control group) in the decomposition of H_2O_2 (to a level higher than 1 mmol/L) were detected between strains isolated from patients with inflammatory bowel disease and from participants in the control group.

peroxide is due to certain *Enterococcus* species rather than the inflammatory conditions at the site of their isolation.

Bacteria belonging to the genera *Streptococcus*, *Lactobacillus* and *Enterococcus* occur in increased numbers in the alimentary tracts patients with IBD and can produce significant amounts of H_2O_2 that can consequently limit the population of anaerobic flora, stimulate the cells of the immune system to release pro-inflammatory cytokines and stimulate the apoptosis of intestinal epithelial cells deprived of their protective mucous layer[36]. Denning *et al*[16] showed that hydrogen peroxide concentrations ranging from 0.5 up to 2 mmol/L were able to induce apoptosis in the cells of the abdominal lining and intestinal epithelium; similar amounts of H_2O_2 were produced *in vitro* by the *Enterococcus* strains examined in this study. These observations support the studies by Kruidenier *et al*[37] and underline the importance of oxidative stress in the pathogenesis of IBD.

During the inflammatory process, the intestinal epithelial cells are exposed to the effects of ROS. If this is a short-term process, no significant damage or disruption of host cell function are observed due to the production of enzymes that inactivate ROS, such as catalase and peroxidase, and DNA repair mechanisms. A significant risk for the intestinal epithelium occurs when ROS are generated at variable concentrations for a longer period of time. Then, not only can the host cells be damaged, but the qualitative and quantitative composition of the specific bacterial species that comprise the commensal flora changes as well[35].

In this work, we showed that enterococci isolated from patients with IBD were able to decompose hydrogen peroxide considerably faster than those isolated from the control group. This may indicate that these strains survived the selective pressures of the inflammatory process. It is likely that only bacterial species possessing a system capable of enzymatic deactivation of ROS were able to survive and subsequently increase their populations on the surface of the damaged intestinal epithelial

cells in patients with IBD. Until recently, bacteria belonging to the genera *Enterococcus*, *Lactobacillus and Pediococcus* were regarded as catalase negative. However, it appears that these bacteria have a very efficient system of hydrogen peroxide deactivation based on manganese-containing catalase or haeme-dependent catalase[38].

In this study, we present data that suggest that processes in the alimentary tract of patients with IBD (most probably based on inflammatory background of this disease) may lead to the selection of certain bacterial species or strains from the constituents of the commensal flora. Our observations have been supported by recent studies on microbiota from biopsies taken from patients with UC and controls performed by Fite *et al*[39], who detected statistically significant differences in the bacterial populations of the UC mucosa and in the control group that varied over the study period. High clinical activity indices and sigmoidoscopy scores were associated with enterobacteria, desulfovibrios, Type E *Clostridium perfringens* and *E. faecalis*. We believe that in case of *Enterococcus*, the strains that adhere strongly to the intestinal epithelium, build three-dimensional biofilm structures and possess enzymatic mechanisms to protect against the effects of ROS produced by the immune cells and other bacterial species have the highest chances of survival.

COMMENTS

Background

Inflammatory bowel disease (IBD) is a chronic inflammation of all or part of the digestive tract. The aetiology of IBD is not fully known, but much attention is currently focused on the role of bacterial flora in the development and/or maintenance of the inflammatory process in patients suffering from this disease. Recent studies indicate that bacteria belonging to the genus *Enterococcus* play an important role in the pathomechanisms underlying IBD based on their virulence potential.

Research frontiers

Changes in the composition of the bacterial flora colonising the human alimentary tract are considered influential in the maintenance of the inflammatory process in patients with IBD. The virulence potential the enterococci may be particularly important. It will be interesting to identify other factors that allow enterococci to develop and maintain the inflammatory process while increasing their chances of survival in unfavourable conditions.

Innovations and breakthroughs

Recent studies have shown that no single aetiological factor could be responsible for the exacerbation of the inflammatory process in IBD. However, an analysis of the quantitative changes in the intestinal microflora of patients with Crohn's disease (CD) demonstrated a significant increase in the population of cocci (including *Enterococcus* and *Streptococcus*) at the sites of inflammation. In the present study, authors observed that virulence genes, such as *asa1* and *gelE*, occurred more frequently in the genomes of *Enterococcus* strains isolated from patients with IBD than in strains isolated from healthy patients. Moreover, authors demonstrated statistically significant differences in the ability of these strains to decompose hydrogen peroxide. Enterococci isolated from patients with IBD decomposed hydrogen peroxide considerably faster than those isolated from the control group. This result indicates that only select bacterial species with an efficient system of enzymatic deactivation of reactive oxygen species (ROS) are able to survive and subsequently increase their populations on the surface of the damaged intestinal epithelial cells.

Applications

The results of this study suggest that *Enterococcus* strains that adhere strongly to the intestinal epithelium, form biofilms and possess enzymatic mechanisms that protect against the effects of ROS have the highest chances surviving and influencing the inflammatory process. Their research improves our understanding of the pathomechanisms underlying IBD.

Peer review

An interesting publication in which authors determine the features of *Enterococcus* that contribute to the development and maintenance of the inflammatory process in patients with IBD. The results are interesting and suggest that *Enterococcus* strains that adhere strongly to the intestinal epithelium, form biofilms and possess antioxidant defence mechanisms seem to have the greatest influence on the inflammatory process.

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ORIGINAL ARTICLE

Aberrant glycosylation of the anti-Thomsen-Friedenreich glycotope immunoglobulin G in gastric cancer patients

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Abstract

AIM: To study whether alterations in the glycosylation of immunoglobulin G (IgG) specific to the Thomsen-Friedenreich glycotope (TF) have diagnostic and prognostic potential in gastric cancer.

METHODS: Serum samples were obtained from patients with histologically verified gastric carcinoma (n = 89), healthy blood donors (n = 40), and patients with benign stomach diseases (n = 22). The lectin-enzyme-linked immunosorbent assay-based glycoprofiling of TF-specific IgG (anti-TF IgG) was performed using synthetic TF-polyacrylamide conjugate as antigen, total IgG purified by affinity chromatography on protein G sepharose, and lectins of various sugar specificities: mannose-specific concanavalin A (ConA), fucose-specific *Aleuria aurantia* lectin (AAL) and sialic acid-specific *Sambucus nigra* agglutinin (SNA). The sensitivity and specificity of the differences between cancer patients and controls were evaluated by receiver operator characteristic (ROC) curve analysis. Overall survival was analyzed by the Kaplan-Meier method. Time-dependent ROC curve statistics were applied to determine cut-off values for survival analysis. All calculations and comparisons were performed using the GraphPad Prism 5 and SPSS 15.0 software.

RESULTS: The level of TF-specific IgG was significantly increased in cancer patients compared with non-cancer controls (P < 0.001). This increase was pronounced mostly in stage 1 of the disease. Cancer patients showed a higher level of ConA binding to anti-TF-IgG (P < 0.05) and a very low level of SNA lectin binding (P = 0.0001). No appreciable stage-dependency of the binding of any lectin to anti-TF IgG was found. A strong positive correlation between the binding of AAL and SNA was found in all groups studied (r = 0.71-0.72; P < 0.0001). The changes in ConA reactivity were not related to those of the fucose- or sialic acid-specific lectin. Changes in the SNA binding index and the ConA/SNA binding ratio demonstrated good sensitivity and specificity for stomach cancer: sensitivity 78.79% (95%CI: 61.09-91.02) and 72.73% (95%CI: 57.21-85.04); specificity 79.17 (95%CI: 65.01-89.53) and 88.64% (95%CI: 71.8-96.6), for the SNA binding index and the ConA/SNA binding ratio, respectively. The other combinations of lectins did not improve the accuracy of the assay. The low level of ConA-positive anti-TF IgG was associated with a survival benefit in cancer patients (HR = 1.56; 95%CI: 0.78-3.09; P = 0.19), especially in stages 3-4 of the disease (HR = 2.17; 95%CI: 0.98-4.79; P = 0.048). A significantly better survival rate was found in all cancer patients with a low reactivity of anti-TF IgG to the fucose-specific AAL lectin (HR = 2.39; 95%CI: 1.0-5.7; P = 0.038).

CONCLUSION: The changes in the TF-specific IgG glycosylation pattern can be used as a biomarker for

stomach cancer detection, and to predict patient survival.

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Key words: Thomsen-Friedenreich antigen; Anticarbohydrate antibodies; Stomach cancer; IgG glycosylation; Survival; Lectins

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INTRODUCTION

The aberrant glycosylation often observed in cancer cells leads to the expression of tumor-associated carbohydrate antigens (TACA) which may be autoimmunogenic and recognized by autoantibodies[1-5]. This makes TACA a promising target for cancer immunotherapy. In cancer patients, an abnormal glycosylation pattern has also been observed for many circulating glycoconjugates, such as transferrin, MUC1 mucin, alpha1-acid glycoprotein, and immunoglobulins[6-10]. This suggests a systemic impact of malignancy on glycosylation machinery or possibly represents a specific feature of the host metabolism. In both cases, such changes might be considered as a biomarker of cancer, a premalignant state, or the disposition of the host to cancer (risk factors).

The Thomsen-Friedenreich antigen (TF, CD176, core-1) (Galβ1, 3GalNAcα/β-O-Ser/Thr) is expressed in many carcinomas and results from incomplete synthesis of O-linked glycans on glycoproteins and glycolipids[1,2]. The TF glycotope is known as a pancarcinoma antigen which is expressed in approximately 90% of all human cancers and in premalignant conditions[2,11]. TF expression is associated with more aggressive tumors and is related to the induction of invasion, metastasis and cancer surveillance mechanisms[12-16]. The TF antigen seems to play a crucial role in the adhesion of cancer cells to the endothelium through interaction with galectin-3, thereby promoting metastasis[17,18].

Naturally-occurring TF antigen-specific immunoglobulin G (anti-TF IgG) autoantibodies are present in human serum in health and disease[3,19,20]. In cancer patients, their level is related to tumor progression and prognosis, being higher in patients with the early stages of the disease, in those with more differentiated tumors (G1-2), and in those with better survival[12,21,22]. This suggests an immediate impact of the humoral immune response on malignancy *via* direct or antibody-dependent cell-mediated effector pathways. However, the mechanisms behind these associations remain to be further elucidated.

Human serum IgG contains N-linked glycans attached to Asn297 on the fragment crystallizable (Fc) region. The Fc glycan structures are highly variable and differ in the level of terminal sialic acid, galactose (G0, G1, G2), core fucose and bisecting GlcNAc[23]. Changes in IgG Fc glycosylation strongly influence the Fc-receptor-mediated activities of antibody[23-25] and are associated with various pathologies, including cancer. However, little attention has been paid yet to the glycosylation of antibodies specific to tumor-associated antigens[26]. During the last decade, the diversity of IgG glycans has been thoroughly studied by the interaction of IgG with lectins[26-29], as well as by mass spectrometry-based methodology[9,10,30]. Our recent studies demonstrated an increase in the level of the ConA lectin-positive glycoform of both total serum IgG and TF antigen-specific IgG in patients with cancer[8,31]. Moreover, the low level of this IgG glycoform was associated with an overall survival benefit in patients with gastric cancer[8], indicating its functional relevance, as well as its potential clinical value. Similar changes in ConA reactivity have been reported for tumor-reactive IgG in patients with ovarian cancer[26]. However, the antigenic specificity of these antibodies remains unknown.

In an attempt to discover and evaluate potential biomarkers for stomach cancer diagnosis and patient prognosis, the TF antigen-specific IgG glycosylation profile was investigated using lectins of various sugar specificities. In this study, we demonstrate the aberrant glycosylation of anti-TF IgG in patients with stomach cancer, and the association of these changes with overall survival, indicating their potential clinical applicability.

MATERIALS AND METHODS

Subjects

Serum samples were obtained from healthy blood donors, patients with benign stomach diseases and patients with histologically verified gastric carcinoma (Table 1). The investigation was carried out in accordance with the ICH GCP Standards and approved by the Tallinn Medical Research Ethics Committee, Estonia. Written informed consent was obtained from each subject.

Tumor staging was based on the histopathological (pTNM) classification of malignant tumors. Serum samples were stored in aliquots at -20 ℃ until use.

Serum IgG purification on protein G sepharose

To remove the anti-TF IgM and IgA isotype antibodies, preliminary purification of serum total IgG was performed on a Protein G HP Spin Trap column as described by the manufacturer (GE Healthcare, United States). The samples were immediately neutralized, dialyzed against phosphate buffered solution (PBS) - 0.1% NaN_3 and stored at + 4 ℃ until tested. About 8.5 mg of IgG was obtained from 1 mL of serum applied onto the Protein G Sepharose column.

Table 1 Characteristics of the subjects tested[1]

Groups	*n*	Male	Female	M/F	Median age (range), yr
Donors	40	12	28	0.43	52.2 (23-70)
Benign group[2]	22	19	3	6.3	62.0 (44-76)
Non-cancer[3]	62	31	31	1	59.5 (23-76)
Cancer patients	89	53	36	1.47	66.0 (22-84)
Stages 1-4					
Stage 1	18	8	10	0.86	65.0 (28-84)
Stage 2	19	14	5	1.67	66.5 (46-83)
Stage 3	42	22	20	0.83	65.0 (38-81)
Stage 4	10	9	1	9	65.0 (44-76)

[1]All subjects were tested for anti-Thomsen-Friedenreich glycotope IgG levels and the concanavalin A binding. *Aleuria aurantia* lectin and *Sambucus nigra* agglutinin binding test was performed in all donors and patients with benign gastric disease, and in 33 patients with stomach cancer (stage 1, $n = 4$; stage 2, $n = 5$; stage 3, $n = 19$; stage 4, $n = 5$); [2]Peptic ulcer disease, $n = 6$; chronic gastritis, $n = 7$; atrophic gastritis, $n = 9$; [3]Combined group of donors and patients with benign stomach disease. M/F: Male/female.

Anti-TF IgG antibody assay

The anti-TF IgG level was determined by enzyme-linked immunosorbent assay (ELISA) as described elsewhere[21], with minor modifications. Briefly, the plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with a synthetic TF-polyacrylamide conjugate (Lectinity, Russia, 10 mol% of carbohydrate) in carbonate buffer, pH 9.6, 5 μg per well. After overnight incubation, triple washing and blocking with a Superblock solution (Pierce, United States) for 15 min at 25 ℃, the purified IgG samples (50 μg/well) in PBS-0.05% Tween (Tw) were applied for 1.5 h at 25 ℃. After subsequent washing with PBS-Tw, the bound anti-TF IgG was detected with alkaline phosphatase conjugated goat anti-human IgG (Dako, United States) and p-nitrophenylphosphate disodium hexahydrate (Sigma, United States). The absorbance values were read at 405 nm (Tecan Reader, Austria). The relatively high doses of total IgG were applied because of the low concentration of anti-TF IgG in the serum. These IgG doses correspond approximately to the 1:25-1:50 serum dilution used in our previous studies[21].

Lectin reactivity of the TF-specific IgG

The lectin reactivity of the TF glycotope-specific IgG was measured in a similar way, except that the binding of mannose-specific concanavalin A (ConA), fucose-specific *Aleuria aurantia* lectin (AAL) and neuraminic acid (sialic acid)-specific *Sambucus nigra* agglutinin (SNA) to the absorbed anti-TF IgG was measured as described by Kodar *et al*[8]. Biotinylated ConA (Sigma, United States) in the ConA binding buffer (0.05 mol/L Tris-HCl buffer, pH 7.2, containing 0.2 mol/L NaCl and 3 mmol/L $CaCl_2$, $MgCl_2$ and $MnCl_2$ each), AAL (Vector Laboratories Inc., United States) in 10 mmol/L HEPES, 0.15 mol/L NaCl buffer, pH 7.5, and SNA (Vector Laboratories Inc., United States) in 10 mmol/L HEPES, 0.15 mol/L NaCl, 0.1 mmol/L $CaCl_2$, pH 7.5, were each applied at a concentration of 5 μg/mL, for 1.5 h at 25 ℃. The bound lectins were detected with a streptavidin-alkaline phosphatase conjugate (Dako, United States) and p-nitrophenylphosphate (Sigma, United States). The optical density value (A) of control wells (blank: the Superblock solution instead of TF-PAA for anti-TF or no sample for lectin binding testing) was subtracted from that of the IgG-coated wells to determine the binding of both IgG and lectin. Each sample was analyzed in duplicate.

To standardize the assay, standard IgG was included in each plate for IgG determination and lectin binding measurement. The interassay variations were minimized by using the correction factor (CF): CF = 1/(standard serum A values - blank) × 100. The results were expressed in relative units (RU): RU = sample A value × CF. The lectin reactivity of anti-TF IgG was calculated as the lectin index: (sample lectin binding RU)/(sample anti-TF IgG binding RU).

Statistical analysis

Comparisons between the groups were performed using the nonparametric Mann-Whitney U test for unpaired data or regression analysis with the Spearman test. Survival analysis was carried out using the Kaplan-Meier method. The receiver operator characteristic (ROC) curve analysis as generated in SPSS 15.0 was used to evaluate the sensitivity and specificity of the changes found for stomach cancer. The area under the ROC curve and the P value of the ROC curve were calculated. The time-dependent ROC curve statistics were applied to determine cut-off values for survival analysis. The difference between the groups was considered to be significant when $P \leqslant 0.05$. All calculations were performed using the GraphPad Prism 5 and SPSS 15.0 software.

RESULTS

TF antigen-specific IgG antibody level in total IgG preparations

A significantly higher level of TF-specific IgG was found in purified total IgG preparations from the serum of patients with stomach cancer compared with that in controls: $P = 0.002$, 0.0003 and 0.00004 for donors, benign and combined non-cancer groups, respectively (Figure 1). This increase was mostly pronounced in stage 1 of the disease ($P = 0.02$, $P = 0.0006$, $P = 0.01$ compared with stages 2, 3 and 4, respectively). Up to 10-fold interindividual variations in anti-TF IgG antibody levels were observed in all the groups and especially in cancer patients.

Lectin binding profile of TF-specific IgG

The anti-TF IgG of patients with cancer showed a significantly higher level of ConA-positive IgG glycoform than that of both controls: $P = 0.013$, 0.05 and 0.005 for donors, benign and non-cancer groups, respectively (Figures 2 and 3). In contrast, the binding of SNA was

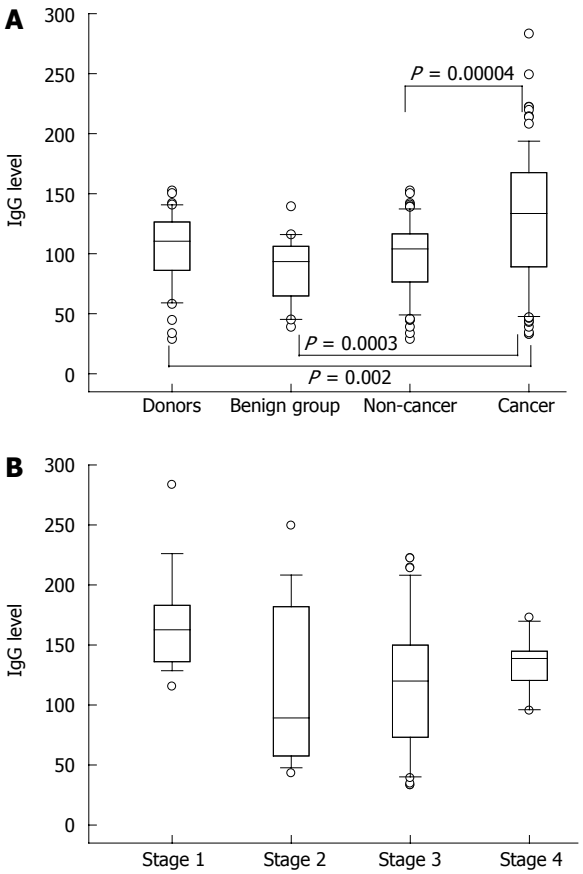

Figure 1 Thomsen-Friedenreich glycotope-IgG level in patients with stomach cancer and controls. A: Box plots of anti-TF IgG levels (medians, ranges and quartiles) in controls and cancer patients; B: Anti-TF IgG levels in cancer patients by stage. Significantly higher in stage 1 compared with stages 2, 3 and 4 (P = 0.02, 0.001 and 0.01, respectively). P values were calculated by the Mann-Whitney U test.

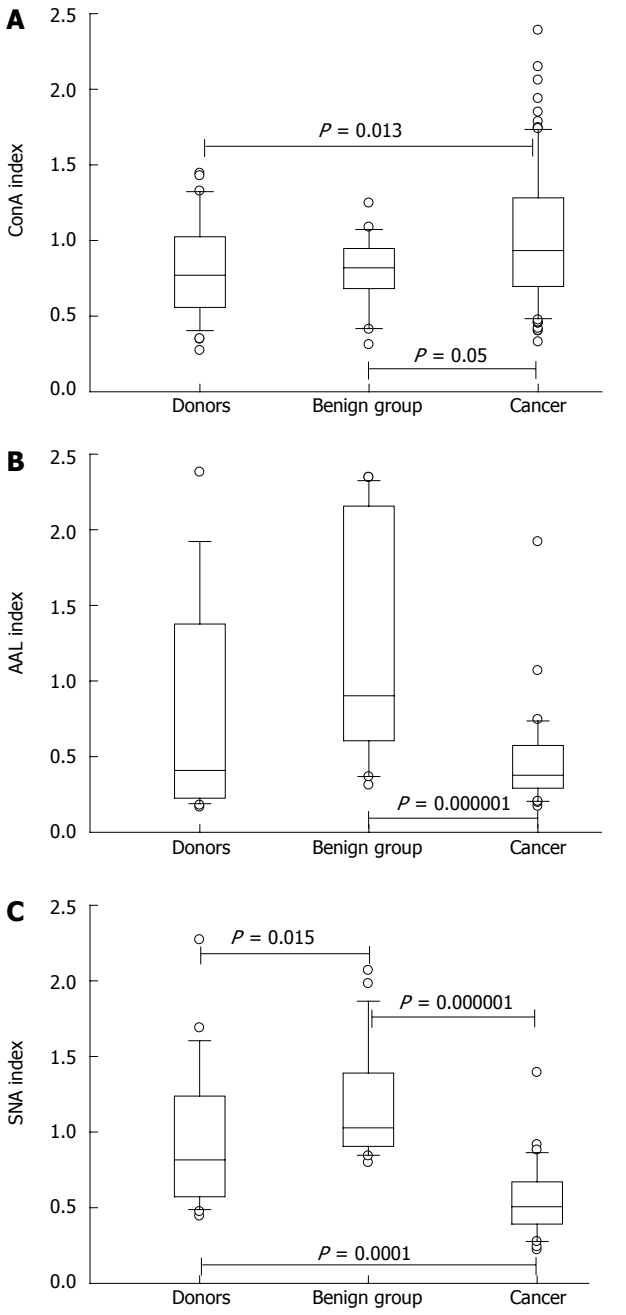

Figure 2 Binding of lectins to the Thomsen-Friedenreich glycotope-specific IgG in gastric cancer patients and controls. A: Concanavalin A (ConA); B: *Aleuria aurantia* lectin (AAL); C: *Sambucus nigra* agglutinin (SNA). Box plots of lectin index values (medians, ranges and quartiles) in patients with stomach cancer, healthy blood donors and patients with benign stomach disease. P values were calculated by the Mann-Whitney U test and are shown for significant differences.

significantly lower in cancer patients compared with that of blood donors and patients with benign gastric diseases ($P < 0.0001$). In cancer patients, the binding of AAL did not differ from that of the donors ($P = 0.64$), but was significantly lower than that of the benign group ($P = 0.000008$) or non-cancer group ($P = 0.005$). A group of patients with chronic gastritis ($n = 7$, one with atrophic gastritis), who showed very high AAL index values, accounted for this difference. This was the only exception in this study when the benign group differed significantly from the donors ($P = 0.01$). Two of these patients also showed a high level of SNA binding. All patients with peptic ulcer disease ($n = 6$) demonstrated a similar level of binding for all three lectins compared with that of blood donors.

No appreciable stage-dependency of the binding of any lectin to the anti-TF IgG was found (Figure 3), though a slight trend towards higher ConA index values in stage 1 cancer patients was observed ($P = 0.19$ compared with stage 3 patients).

A strong positive correlation between the reactivities of AAL and SNA was demonstrated in all groups: cancer patients ($r = 0.72$; $P < 0.0001$); non-cancer group ($r = 0.71$; $P < 0.0001$) as well as the combined group of all tested subjects ($r = 0.72$; $P < 0.0001$). No significant correlation between the reactivities of ConA and the two other lectins was observed. Thus, the changes in ConA reactivity were not related to the modification of anti-TF IgG binding sites for the fucose- or sialic acid-specific lectins (AAL and SNA).

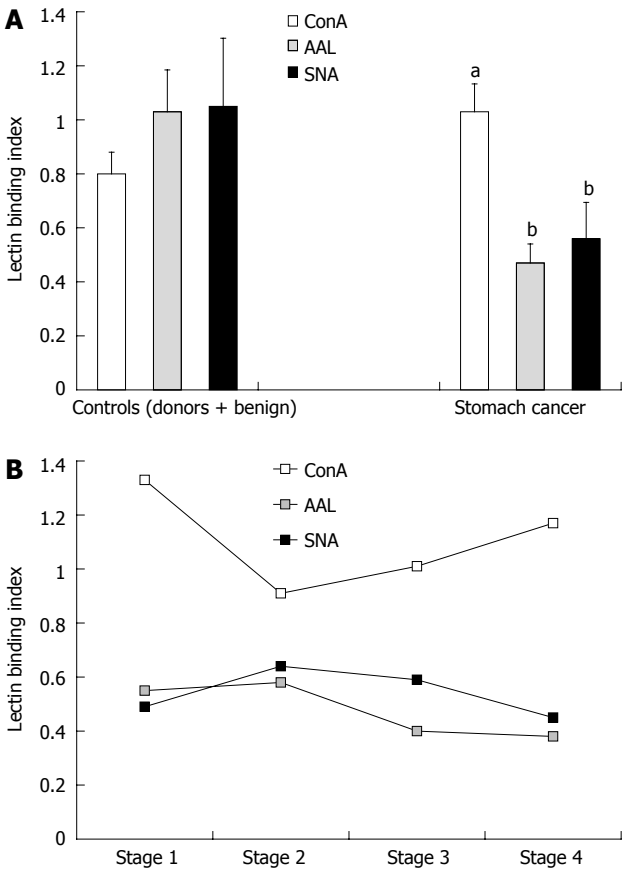

Figure 3 Anti-Thomsen-Friedenreich glycotope-specific IgG glycosylation profile as defined by concanavalin A, *Aleuria aurantia* lectin and *Sambucus nigra* agglutinin binding. A: Non-cancer group and cancer patients; B: Relation to the stage of cancer. The results are depicted as mean with error bars representing SEM. *P* values were calculated by the Mann-Whitney *U* test: $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* controls for all lectins. ConA: Concanavalin A; AAL: *Aleuria aurantia* lectin SNA: *Sambucus nigra* agglutinin.

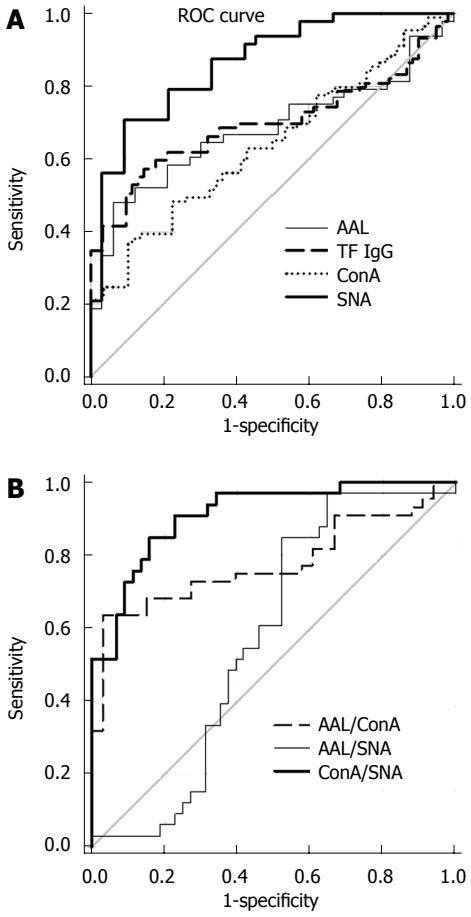

Figure 4 Sensitivity and specificity of anti-Thomsen-Friedenreich glycotope-specific IgG glycosylation changes in stomach cancer. A: Receiver operator characteristic (ROC) curve analysis by lectin binding index values; B: ROC curve analysis by lectin combinations using the ratios of concanavalin A (ConA)/*Sambucus nigra* agglutinin (SNA), ConA/*Aleuria aurantia* lectin (AAL) and AAL/SNA.

Anti-TF IgG level and lectin binding profile: Sensitivity and specificity in stomach cancer

ROC curve analysis was used to evaluate changes in the level and glycosylation profile of anti-TF-IgG as possible biomarkers. The diagnostic accuracy and ROC curve statistics are presented in Figure 4 and Table 2. In the absence of a correlation between the binding of ConA and the two other lectins, we investigated a possible interactive effect of lectin combinations using the ratios of ConA/SNA, ConA/AAL and AAL/SNA in the ROC analysis.

Despite the significant difference in anti-TF IgG levels between cancer patients and controls these changes showed a low sensitivity and specificity for cancer, possibly due to great variations within each group. The same was true for the ConA and AAL lectin index values. In contrast, changes in the SNA binding index and, especially, ConA/SNA ratio values demonstrated rather good sensitivity and specificity reaching 78.8%-88.6% (Table 2). Since no notable cancer stage dependency of lectin binding was observed, the sensitivity and specificity values are presented for the combined cancer group and non-cancer controls (Figure 4 and Table 2). Using the combination of ConA/AAL and AAL/SNA lectin ratios did not improve the accuracy of the assay and showed lower sensitivity and specificity values, usually below 70% (Table 2).

Lectin binding of TF-specific IgG and survival of cancer patients

The subgroups of cancer patients with high and low levels of lectin binding to anti-TF IgG were compared. The cut-off levels were calculated using time-dependent ROC curve analysis for each lectin. Despite the opposite changes in the binding of ConA and AAL or SNA lectin in cancer patients (increase *vs* decrease) a similar association with survival was demonstrated for all three lectins tested, with a common trend in the early and advanced stages of the disease (Figure 5).

The low level of ConA-positive anti-TF IgG was associated with a survival benefit in cancer patients (HR = 1.56; 95%CI: 0.78-3.09; $P = 0.19$), especially in those with stages 3-4 of the disease (HR = 2.17; 95%CI: 0.98-4.79; $P = 0.048$). A significantly better survival rate was found in all cancer patients with low reactivity

Table 2 Anti-TF IgG level and lectin binding profile: Sensitivity and specificity for gastric cancer

Parameter	Sensitivity % (95%CI)	Specificity % (95%CI)	ROC statistics		Sensitivity at specificity 90%
			Area under curve	*P* value	
Anti-TF IgG	65.17 (51.34-76.26)	67.74 (56.66-76.98)	0.70	< 0.0001	50.56
ConA	62.92 (48.37-74.49)	56.90 (46.37-67.74)	0.64	0.004	24.72
SNA	78.79 (61.09-91.02)	79.17 (65.01-89.53)	0.87	< 0.0001	57.58
AAL	69.70 (51.29-84.41)	64.58 (49.46-77.84)	0.68	0.006	12.12
ConA/SNA	72.73 (57.21-85.04)	88.64 (71.80-96.60)	0.91	< 0.0001	63.64
AAL/ConA	84.85 (68.10-94.89)	68.18 (52.42-81.39)	0.78	< 0.0001	33.33
AAL/SNA	84.85 (72.24-93.93)	47.92 (30.80-66.46)	0.68	0.006	3.03

ConA: Concanavalin *A*; AAL: *Aleuria aurantia* lectin; SNA: *Sambucus nigra* agglutinin; Anti-TF IgG: Anti-Thomsen-Friedenreich glycotope immunoglobulin G; ROC: Receiver operator characteristic.

of anti-TF IgG to the fucose-specific AAL lectin (HR = 2.39; 95%CI: 1.0-5.7; $P = 0.038$). The association of SNA lectin reactivity with survival showed a similar trend. Considering that no correlation between the binding of ConA and the two other lectins was found, a possible interactive effect of the combination of the two lectins was investigated using the ratios of ConA/SNA, ConA/AAL and AAL/SNA. However, no additional information regarding association with survival was obtained (data not shown).

DISCUSSION

The role of autoantibodies against tumor-associated glycans in cancer surveillance has been mostly considered for IgM[2,4]. These antibodies are not affinity-matured which argues in favor of their inherent natural origin. In contrast, the presence of IgG anti-glycan antibodies suggests an adaptive immune response. The origin of anti-glycan autoantibodies of both isotypes is still unclear though the available evidence suggests that at least anti-TF and anti-alpha Gal antigen-specific antibodies may be induced by bacterial glycans or, possibly, by cross-reactivity with these antigens[3,32]. In any case, large and unexplained interindividual variations in their level in health and disease exist[21,22], possibly reflecting the distinct immunological histories of each individual. Moreover, the anti-TF IgG level is rather stable over time at an individual level in both patients and controls[22,33].

In this study, a significantly higher level of TF-specific IgG in purified total IgG preparations from the serum of patients with stomach cancer than in both control groups was observed. This increase was mostly pronounced in stage 1 of the disease, suggesting that an adaptive immune response cannot be excluded in the early stages of tumor with a subsequent decrease in the anti-TF IgG level in advanced cancer as a result of tumor-induced immunodepression. If this is the case, the population of anti-TF IgG should be expected to be heterogeneous and to include both naturally-occurring TF glycotope-specific antibodies, whose presence precedes tumor development, and those triggered by disease, *i.e.*, induced by the tumor-derived TF glycotope. Because of large interindividual variations in anti-TF IgG level, the human population may be divided into low and strong responders to the TF glycotope. Notably, donors and the benign group showed a more compact distribution. Further characterization of anti-TF IgG subpopulations is needed to determine their structural and functional diversities, and clinical significance.

A significantly higher binding of ConA ($P = 0.005$) and a highly significant ($P = 0.00000003$) decrease in SNA lectin binding was characteristic of the anti-TF IgG from the samples of cancer patients compared with those of the non-cancer group (Figure 3). The ConA binding index was higher in stage 1 patients, whereas the SNA and AAL lectin binding index values were low irrespective of the disease stage. We previously found similar changes in the binding of ConA to the total IgG from the serum of patients with gastric cancer.

The changes in anti-TF IgG glycosylation showed rather a high level of sensitivity and specificity in cancer and non-cancer group discrimination (Table 2). It appears that the SNA binding index and, in particular, the ConA/SNA ratio are promising as diagnostic markers to differentiate stomach cancer from controls, including benign gastric diseases. As there are no reliable markers for gastric cancer yet, these findings may be of clinical value.

We reported recently that the level of agalactosylated IgG (G0 glycoform) in the total IgG of gastric cancer patients was significantly higher than that of controls[9]. Interestingly, this shift positively correlated with the binding of ConA to the total serum IgG, and was also observed in purified TF-specific IgG samples (unpublished data). This indicates that IgG asialylation/agalactosylation is associated with an increased ConA binding possibly due to a better accessibility of the d-mannose residue to the ConA because of conformational changes in the Fc G0 glycoform. The absence of a correlation between the binding of ConA and SNA may be a reason for a positive interactive effect of using the ConA/SNA binding ratios for cancer *vs* non-cancer group discrimination.

Despite the opposite changes in the binding of ConA and the two other lectins to anti-TF IgG in pa-

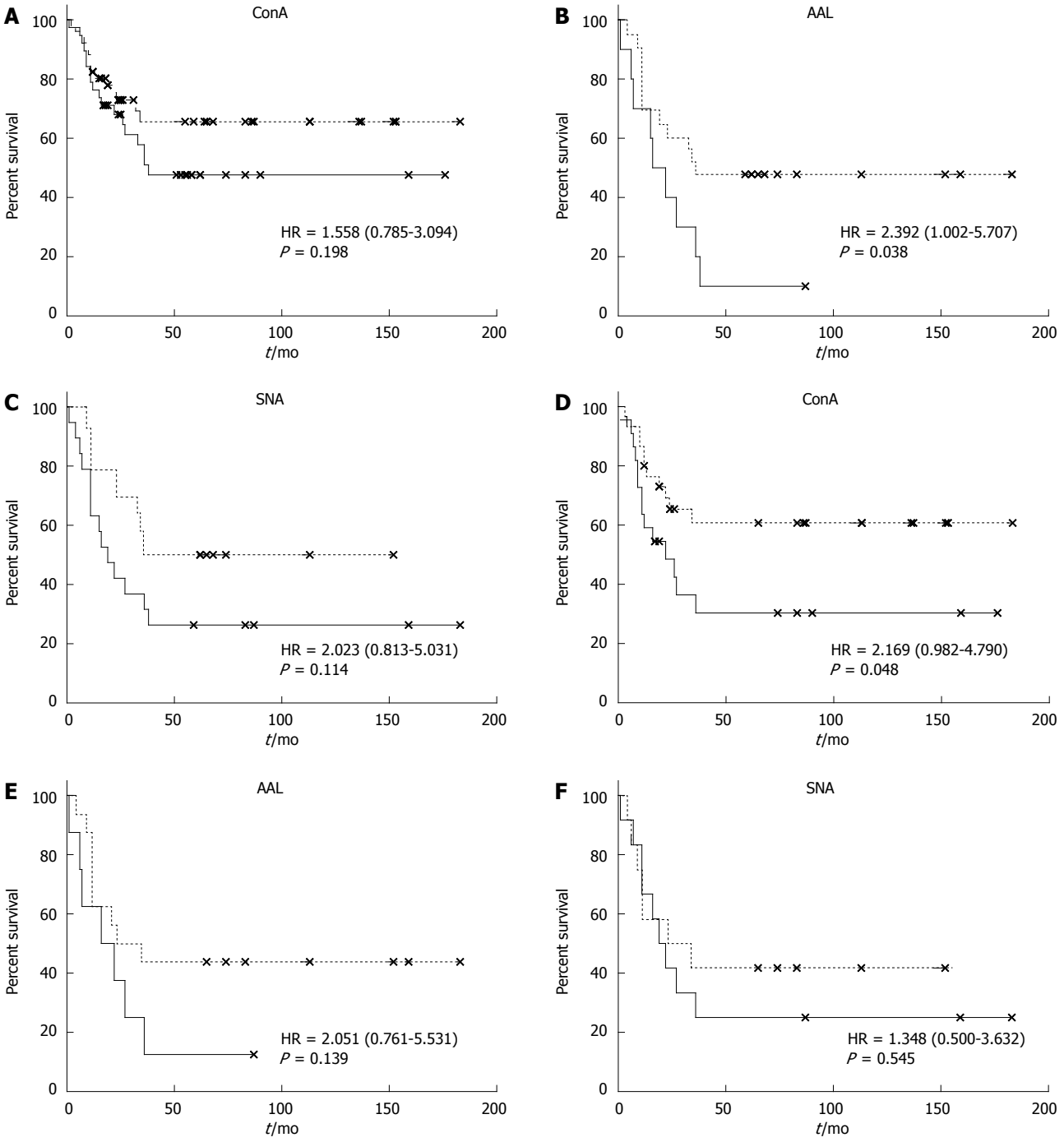

Figure 5 Binding of lectins to Thomsen-Friedenreich glycotope-specific IgG and the survival of patients with stomach cancer. The probability of survival (Kaplan-Meier method) of stomach cancer patients in relation to lectin binding to Thomsen-Friedenreich glycotope (TF)-specific IgG. A: Concanavalin A (ConA) lectin binding of all cancer patients; B: *Aleuria aurantia* lectin (AAL) binding in all cancer patients; C: *Sambucus nigra* agglutinin (SNA) binding in all cancer patients; D: ConA binding in patients with stage 3-4 cancer; E: AAL binding in patients with stage 3-4 cancer; F: SNA binding of patients with stage 3-4 cancer. Patient lectin index values, which were either lower, equal to (dashed line) or higher (solid line) than the cut-off are compared. The cut-off levels were calculated using time-dependent receiver operator characteristic curve analysis.

tients with cancer (Figure 2), a better survival rate was associated with lower reactivity of anti-TF IgG to AAL and ConA. The SNA reactivity revealed no significant association with survival, though a similar slight trend was observed. It seems that IgG desialylation alone is not sufficient for the impact on survival, and further degalactosylation is needed to attain this effect. In this study, the patients were subjected to follow-up for more than 10 years. The association of the binding of ConA and AAL with survival became evident after 2.5-3 years of observation, reaching a maximum after 5 years.

Several studies have demonstrated that agalactosylated IgGs show an increased inflammatory activity[34-37] that may promote tumor growth[38,39]. In addition, the IgG_1 lacking the branching fucose or with an additional bisecting GlcNAc shows an enhanced ADCC activity

through an increased interaction with Fc gamma receptors[36,40-42]. The association of the high level of the G0 IgG glycoform with a lower survival rate we reported recently[9], and the decreased anti-TF IgG fucosylation associated with better survival in the present findings may be related to these mechanisms.

It has been shown that sialylated glycans predominate in glycosylated antibody binding fragments (Fab) whereas the Fc fragment N-glycans are mostly monosialylated[30,43]. Our findings cannot answer the question of whether the Fc or Fab fragment sialylation is responsible for the changes observed in anti-TF IgG glycosylation.

In addition, it remains unclear whether changes in the sialylation and core fucosylation of anti-TF IgG glycans in both IgG fragments may be independent or concordant events, despite the positive correlation observed between the binding of SNA and AAL lectin to the whole molecule of the TF-specific IgG. Given that an active immunization reduces the sialylation of IgG, especially the antigen-driven IgG[36], we hypothesize that the decreased sialylation of anti-TF IgG observed in cancer patients may be an indicator of an adaptive immune response to tumor-derived TF antigen, in addition to naturally-occurring anti-TF IgG antibodies, which are present in every individual in different amounts.

In conclusion, the results show the glycosylation of TF-specific IgG antibodies in patients with gastric cancer to undergo significant changes when compared with that of controls. The appearance of these alterations already in the early stages of cancer and their association with survival suggest that they play a significant role in cancer development and progression. The lectin-based glycoprofiling ELISA assay is an informative and clinically applicable tool for the analysis of IgG glycans. The results imply that changes in the TF-specific IgG glycosylation have diagnostic and prognostic potential for stomach cancer. However, a further study is needed to support these findings on a larger scale using different control groups. Mass spectrometry-based methodology might help to further specify different subsets of anti-TF IgG of clinical importance.

COMMENTS

Background

Gastric cancer is the second leading cause of cancer deaths worldwide. Yet there are still no reliable serum biomarkers for gastric cancer diagnostics and prognostics. Previous studies have demonstrated that naturally-occurring antibodies (Ab) to tumor-associated glycans are involved in natural tumor immunity, being associated with tumor progression and cancer patients survival.

Research frontiers

Recent findings about the impact of Ab glycosylation on their effect or functions suggest that the evaluation of not just the level of antibodies but rather their structural diversity might improve the clinical potential of the antibody-based approach in the disease diagnostics and prognostics. With this purpose in mind, a research team from Estonia led by Kurtenkov O aimed to evaluate whether the glycosylation profile of immunoglobulin G (IgG) antibody to the so-called tumor-associated Thomsen-Friedenreich antigen (TF) could serve as a marker of gastric cancer and the disease outcome. The study is based on the analysis of a long-term (for more than 10 years) survival of cancer patients.

Innovations and breakthroughs

This study demonstrates for the first time that the glycosylation of anti-TF antibodies reveals gastric cancer-related changes with a diagnostic sensitivity and specificity of about 80%. It is striking that the stage of cancer had little effect on these parameters, thus allowing one to diagnose the disease in its early stages. Besides, the authors show that some Ab glycoforms may predict patient survival. Based on these results, the authors conclude that this serology-based approach may be of clinical importance for gastric cancer diagnostics and prognostics.

Applications

The results indicate that changes in the TF-specific IgG glycosylation have diagnostic and prognostic potential for stomach cancer. The lectin-based glycoprofiling of anti-TF IgG antibodies is an informative and clinically applicable tool to specify sets of tumor-associated antibody glycoforms which can be used as a biomarker for stomach cancer detection and follow-up. It is plausible that the elevated level of a specific antibody glycoform might serve as a marker for a stronger immune response, providing protection against cancer. The findings may eventually lead to the development of novel forms of cancer immunotherapy based on vaccination with TF as target and the manipulation of Ab glycosylation machinery. This will need further work, in particular performance of functional tests using tumor cells and different Ab glycoforms, to understand the biological relevance of the results.

Terminology

Glycans: The chains of sugars that coat the outer surface of cells or are attached to other molecules (proteins, lipids). Glycosylation is the enzymatic process in which sugars are attached to other molecules.

Peer review

This important study extends findings from studies of the same authors published previously and make a great contribution to the understanding of the molecular mechanisms that underline aberrant glycosylation in gastric carcinogenesis, and to the evaluation of potential biomarkers for gastric cancer diagnosis and patient prognosis.

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ORIGINAL ARTICLE

Bone-marrow mesenchymal stem cells reduce rat intestinal ischemia-reperfusion injury, ZO-1 downregulation and tight junction disruption *via* a TNF-α-regulated mechanism

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Author contributions: Song HL and Shen ZY designed the research, analyzed and interpreted the data, and wrote the manuscript; Shen ZY, Zheng WP and Zhang J performed the research; Song HL and Zheng WP analyzed the data; all authors have read and approved the final manuscript.

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Abstract

AIM: To investigate the effect of bone-marrow mesenchymal stem cells (BM MSCs) on the intestinal mucosa barrier in ischemia/reperfusion (I/R) injury.

METHODS: BM MSCs were isolated from male Sprague-Dawley rats by density gradient centrifugation, cultured, and analyzed by flow cytometry. I/R injury was induced by occlusion of the superior mesenteric artery for 30 min. Rats were treated with saline, BM MSCs (*via* intramucosal injection) or tumor necrosis factor (TNF)-α blocking antibodies (*via* the tail vein). I/R injury was assessed using transmission electron microscopy, hematoxylin and eosin (HE) staining, immunohistochemistry, western blotting and enzyme linked immunosorbent assay.

RESULTS: Intestinal permeability increased, tight junctions (TJs) were disrupted, and zona occludens 1 (ZO-1) was downregulated after I/R injury. BM MSCs reduced intestinal mucosal barrier destruction, ZO-1 downregulation, and TJ disruption. The morphological abnormalities after intestinal I/R injury positively correlated with serum TNF-α levels. Administration of anti-TNF-α IgG or anti-TNF-α receptor 1 antibodies attenuated the intestinal ultrastructural changes, ZO-1 downregulation, and TJ disruption.

CONCLUSION: Altered serum TNF-α levels play an important role in the ability of BM MSCs to protect against intestinal I/R injury.

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Key words: Bone marrow mesenchymal stem cells; Zona occludens 1; Ischemia-reperfusion injury; Intestinal mucosa; Tumor necrosis factor-α

Core tip: Intestinal ischemia/reperfusion (I/R) injury is clinically important. Bone-marrow mesenchymal stem cells (BM MSCs) can protect against I/R injury; however, the mechanism is unclear. This study demonstrates that submucosal infusion of BM MSCs decreased intestinal permeability and preserved intestinal mechanical barrier function after I/R injury in rats, *via* a mechanism linked to reduced serum tumor necrosis factor (TNF)-α levels and increased expression of the intestinal tight junction protein zona occludens 1. Altered serum TNF-α levels play an important role in the ability of BM MSCs to protect against intestinal I/R injury.

Shen ZY, Zhang J, Song HL, Zheng WP. Bone-marrow mesenchymal stem cells reduce rat intestinal ischemia-reperfusion injury, ZO-1 downregulation and tight junction disruption *via* a TNF-α-regulated mechanism. *World J Gastroenterol* 2013;

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INTRODUCTION

Digestive organ transplantation and other abdominal surgical procedures can result in different degrees of intestinal ischemia/reperfusion (I/R) injury, which can delay patient recovery and lead to systemic organ failure. Therefore, intestinal I/R injury is an important clinical issue. The small intestine is composed of labile cells that are easily injured by I/R; however, the mechanisms responsible for intestinal I/R injury are unclear. Previous studies have reported that the serum level of tumor necrosis factor (TNF)-α is elevated in patients with severe intestinal I/R injury[1]. TNF-α is a cytokine with broad-spectrum physiological and pathological responsiveness, which is primarily secreted by monokaryons and macrophages. In addition to participating in the humoral and cellular immune responses, TNF-α also plays an important role in diseases such as severe hepatitis, septic shock and inflammatory bowel disease[2-6]; however, it is not known whether TNF-α affects the intestinal barrier function during I/R injury.

Bone-marrow mesenchymal stem cells (BM MSCs) are fibroblast-like, pluripotent adult stem cells. BM MSCs can adhere to plastic and grow readily in the laboratory. BM MSCs give rise to mesoderm cells[7,8], and have been reported to differentiate into all three germ cell lines[9], liver and neural cells[10,11], which have potential to be used for the treatment of various diseases. Allogeneic MSCs were transplanted into primates *via* an intravenous route and distributed to the gastrointestinal tract where they proliferated[12]. MSCs have also been shown to have immunomodulatory capabilities due to the secretion of several growth factors[13,14]. BM MSCs reduce intestinal I/R injury in rats[15]. Studies in I/R rodent models have demonstrated that MSCs can beneficially produce paracrine growth factors and anti-inflammatory cytokines[16]. It should be noted that MSCs respond to TNF-α, but do not produce TNF-α[17].

The intestinal mucosa is the physical and metabolic barrier against toxins and pathogens in the gut lumen. Tight junctions (TJs) are the main structures responsible for restricting the paracellular movement of compounds across the intestinal mucosa. Structurally, TJs are composed of cytoplasmic proteins, including the zona occludens proteins, ZO-1-3[18,19] and two distinct transmembrane proteins, occludin and claudin[20,21], which are linked to the actin-based cytoskeleton[22]. TJs function as occlusion barriers by maintaining cellular polarity and homeostasis, and by regulating the permeability of paracellular spaces in the epithelium[23]. ZO-1, a member of the membrane-associated guanylate kinase family of proteins, acts as a scaffold for the organization of transmembrane TJ proteins, and also recruits various signaling molecules and the actin cytoskeleton to TJs[24]. Although previous studies have provided an insight into the molecular structure of TJs, much less is known about TJ functionality under physiological or pathophysiological conditions. Few studies have described the intestinal mucosa ultrastructure or changes in TJs during I/R injury.

In this study, we used a rat model of intestinal I/R injury to investigate the effect of BM MSCs on intestinal mucosa ultrastructure, with an emphasis on the mechanisms of intestinal barrier dysfunction.

MATERIALS AND METHODS

Animals and I/R injury model

Male Sprague-Dawley rats (180-200 g) were obtained from the Military Medical Science Academy of China People's Liberation Army (PLA; Beijing, China), housed at a constant temperature and humidity, and provided with food and water *ad libitum*. All animal experimental procedures were approved by the Ethics Committee of the Military Medical Science Academy of the PLA before commencement of the study.

One-hundred and eight male rats were fasted for 12 h with free access to water before surgery and randomly assigned to five experimental groups. The operative procedures were performed using standard sterile technique under general anesthesia using 5% chloral hydrate (10 mL/kg). All rats were subjected to laparotomy using a midline incision that was approximately 3 cm, and the principal branches of the superior mesenteric artery (SMA) were identified. In the Sham group, the SMA was isolated using blunt dissection, without clamping the vessel. In the BM MSCs + I/R injury group, the SMA was occluded for 30 min using an atraumatic microvascular clamp. Immediately after the clamp was released, 1 × 10^7 male rat BM MSCs suspended in 0.5 mL serum-free Dulbecco's Modified Eagle's Medium (DMEM) were injected into the intestinal submucosa at five different locations. Animals in the normal saline (NS) + I/R injury group underwent I/R followed by the injection of 0.5 mL normal saline into the intestinal submucosa at 10 different locations. The anti-TNF-α + I/R injury group and the anti-TNF-αR1-IgG + I/R injury group were administered with anti-TNF-α IgG (1000 μg per rat; United States Biological, Swampscott, MA, United States) or anti-TNF-α R1 antibody (1000 μg per rat; R and D Systems, Minneapolis, MN, United States), respectively. Injections were given *via* the tail vein after induction of I/R injury.

The abdomen was closed and the animals were allowed to recover with free access to tap water and standard pellet rat chow. Rats in the I/R injury, BMSCs + I/R injury and Sham groups were euthanized at 2, 6, 24, 72 and 144 h after I/R injury (n = 6 at each time point). Rats in the anti-TNF-α IgG + I/R and anti-TNF-α R1

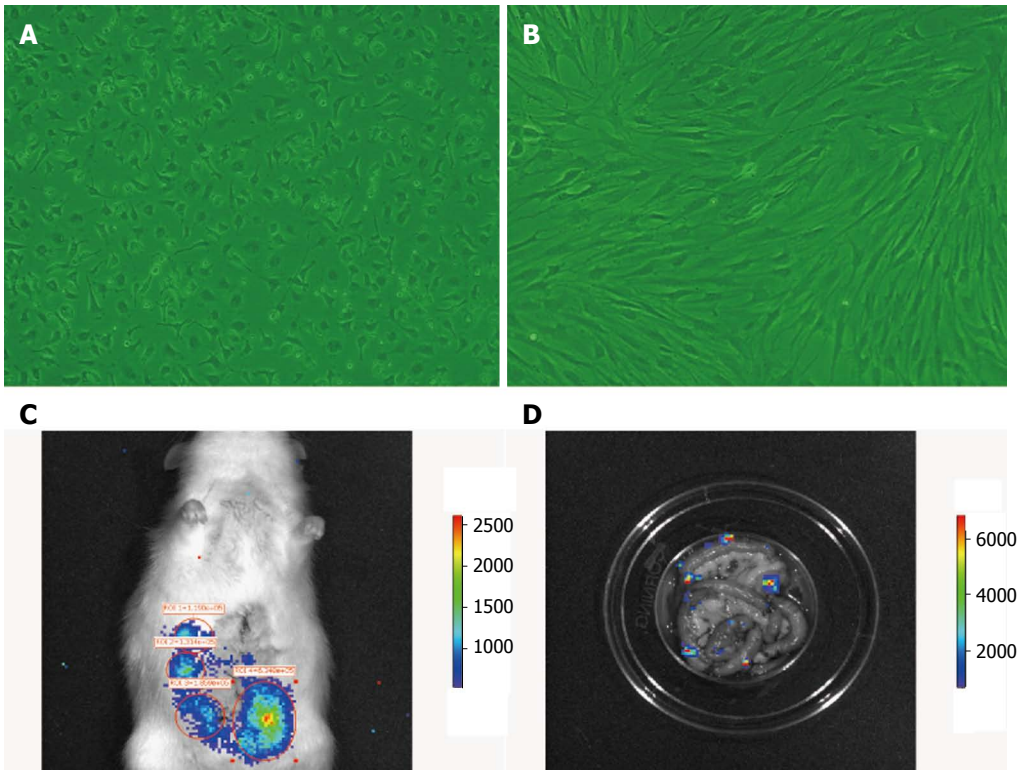

Figure 1 Morphology of bone-marrow mesenchymal stem cells *in vitro*, and *in vivo* cell tracing of bone-marrow mesenchymal stem cells colonization in rat intestine. A: First-passage bone-marrow mesenchymal stem cells (BM MSCs); B: Third-passage BM MSCs (× 200); C: Homing of fluorescently labeled BM MSCs (B16-F10-Luc-G5) to the rat intestine 6 h after transplantation; D: After the intestine was removed and washed repeatedly, fluorescently labeled BM MSCs were still observed, confirming the cells homed to the intestine and survived.

antibody + I/R injury groups were euthanized at 6 h after I/R injury (n = 6 each). Blood samples and approximately 5 cm of the ileum were collected from each rat. The plasma was separated by centrifugation and stored at -80 ℃ until analysis. The intestinal samples were fixed for histopathological analysis and transmission electron microscopy.

Isolation and characterization of BM MSCs

BM MSCs were isolated from the femur and tibia of male Sprague-Dawley rats (100-120 g). Red blood cells were lysed using 0.1 mol/L NH_4Cl, and the remaining cells were washed, resuspended, and cultured for 4 wk in DMEM/F12 (Gibco, Carlsbad, CA, United States) containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 15% fetal bovine serum. BM MSCs were cultured in an incubator at 37 ℃, 5% CO_2 with saturated humidity. The medium was changed every 72 h.

When the third-passage cells reached 80% confluence, the cells were trypsinized, washed, centrifuged, and resuspended at 1 × 10^7 cells/mL in phosphate-buffered saline (PBS). BM MSCs were stained using antibodies against CD29, CD90, RT1A, CD45 and RT1B (Biolegend, San Diego, CA, United States) and CD34 (Santa Cruz Biotechnology, Santa Cruz, CA, United States). They were analyzed by flow cytometry (FACSCalibur; BD, Alaska, MN, United States). The proportion of $CD29^-$, $CD90^-$ and RT1A-positive cells, and $CD34^-$, $CD45^-$ and RT1B-negative cells was > 98% (Figure 1A). BM MSCs were also confirmed as plastic-adherent cells with a spindle-shaped morphology under standard culture conditions by microscopy (Figure 1B). The purity of BM MSCs was > 95%.

Detection of donor BM MSCs in recipient intestines

BM MSCs (1 × 10^7 cells) were incubated with 3.5 μg/mL 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indoctricarbocyanine Iodide (DIR) in 10 mL PBS containing 0.5% ethanol for 30 min at 37 ℃, and washed twice with PBS. Sprague-Dawley rats weighing 180-200 g were anesthetized using 5% chloral hydrate, subjected to a midline laparotomy, and 1 × 10^7 labeled BM MSCs suspended in 1 mL PBS were injected into the intestinal submucosa at five different points. At 2, 6, 24, 72 and 144 h later, luciferin was injected abdominally using a 25-gauge needle and, 7-8 min later, the animals were anesthetized and imaged using a high-sensitivity optical molecular imaging and high-resolution digital X-ray system (IVIS Lumina II, Alameda, CA, United States).

Histological measurement of intestinal mucosal injury

Serial 2-cm samples were taken from the terminal ileum and fixed with 10% neutral formalin. Tissues were processed, embedded, and stained with hematoxylin and eosin. Three paraffin sections were prepared from each tissue sample. Two pathologists who were blinded to the

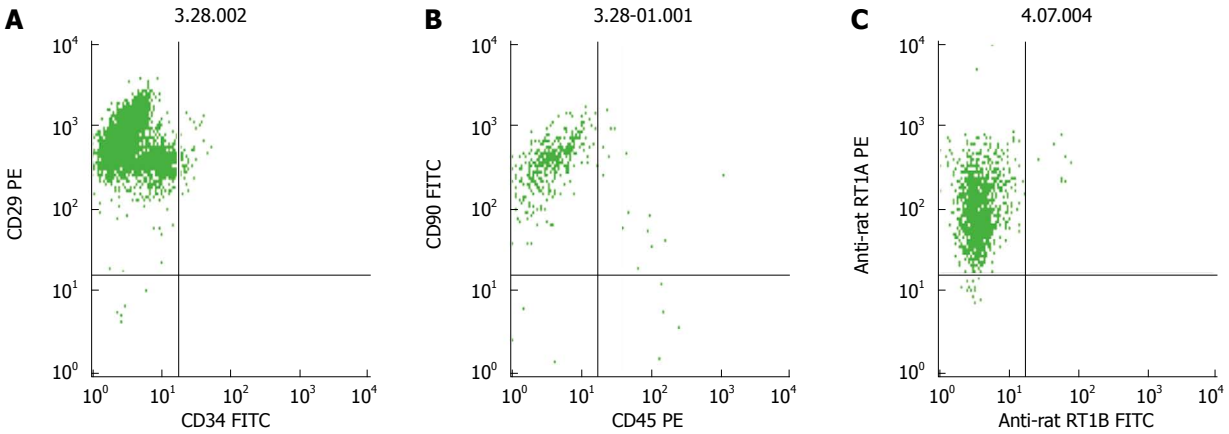

Figure 2 Flow cytometric analysis of third-passage bone-marrow mesenchymal stem cells. A: The proportion of CD29-positive and CD34-negative cells was approximately 96%; B: The proportion of CD90-positive and CD450-negative was approximately 98%; C: The proportion of RT1A-positive and RT1B-negative cells was > 98%.

source of the slides analyzed. The degree of histopathological changes was graded semiquantitatively using the histological injury scale previously described by Chiu *et al*[25], as follows: 0, normal mucosal villi; 1, development of a subepithelial space, usually at the apex of the villi with capillary congestion; 2, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; 3, massive epithelial lifting down the sides of the villi and ulceration at the villous tips; 4, denuded villi with dilated capillaries and increased cellularity of the lamina propria; and 5, degradation and disintegration of the lamina propria, hemorrhage, and ulceration. A minimum of six randomly chosen fields from each rat were evaluated and averaged to determine the degree of mucosal damage.

Serum D-lactate, diamine oxidase and TNF-α assay

The serum levels of TNF-α, D-lactate and diamine oxidase (DAO) were determined using enzyme linked immunosorbent assay kits (R and D Systems) according to the manufacturer's protocol.

Detection and observation of intestinal mucosal ultrastructure

Ultrathin (70-nm) intestinal sections were prepared using standard techniques and examined using a transmission electron microscope (Hitachi H-600, Tokyo, Japan).

Immunohistochemical detection of ZO-1 in frozen tissue sections

Frozen intestinal tissue sections (5 μm) were fixed on glass slides by incubation in acetone for 10 min at 4 ℃, and then incubated with 3% H_2O_2 for 20 min at room temperature, blocked in goat serum for 30 min at 37 ℃, and then indirectly immunolabeled with a rabbit anti-mouse polyclonal ZO-1 antibody (1:50; Santa Cruz Biotechnology) using an ABC kit at 4 ℃ overnight (Takara, Dalian, China), according to the manufacturer's instructions. For the negative controls, the primary antibody was replaced with PBS. The sections were then incubated in biotinylated goat anti-rabbit IgG (1:300 in PBS; Histostain-Plus kit, Zymed Laboratories, South San Francisco, CA, United States) for 2 h at room temperature, rinsed in PBS, rinsed in distilled water, then the staining was developed using 3,3'-diaminobenzidine and the sections were counterstained using hematoxylin.

Western blotting of tissue ZO-1 content

Intestinal tissue samples were homogenized in lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 1% Triton × 100, 0.2 mol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 1 mol/L DTT and 2 mol/L aprotinin]. The protein samples (50 μg) were electrophoresed on 8% SDS-PAGE gels, transferred to nitrocellulose membranes, blocked with non-fat dried milk in TBS containing 0.05% Tween-20 (TTBS) for 1 h at room temperature, and incubated with a rabbit anti-mouse polyclonal ZO-1 antibody (1:400; Santa Cruz Biotechnology) at 4 ℃ overnight. After three washes in TTBS, the membranes were incubated with alkaline-phosphatase-labeled goat anti-rabbit IgG (1:2000; Santa Cruz Biotechnology) for 2 h at room temperature. The bands were visualized using α-dianisidine and β-naphthyl acid phosphate (Sigma, St Louis, MO, United States).

Statistical analysis

SPSS version 10.0 (SPSS, Chicago, IL, United States) was used for the statistical analysis. Normally distributed data were shown as the mean ± SD. Different groups of data were compared by analysis of variance (ANOVA). The degree of relationship between TNF-α and the Chiu risk score was evaluated by a bivariate correlation. The results was statistically significant when $P < 0.05$, and was highly significant when $P < 0.01$.

RESULTS

Culture of BM MSCs

The cells were confirmed as BM MSCs based on their spindle-shaped morphology, adherence to plastic (Figure

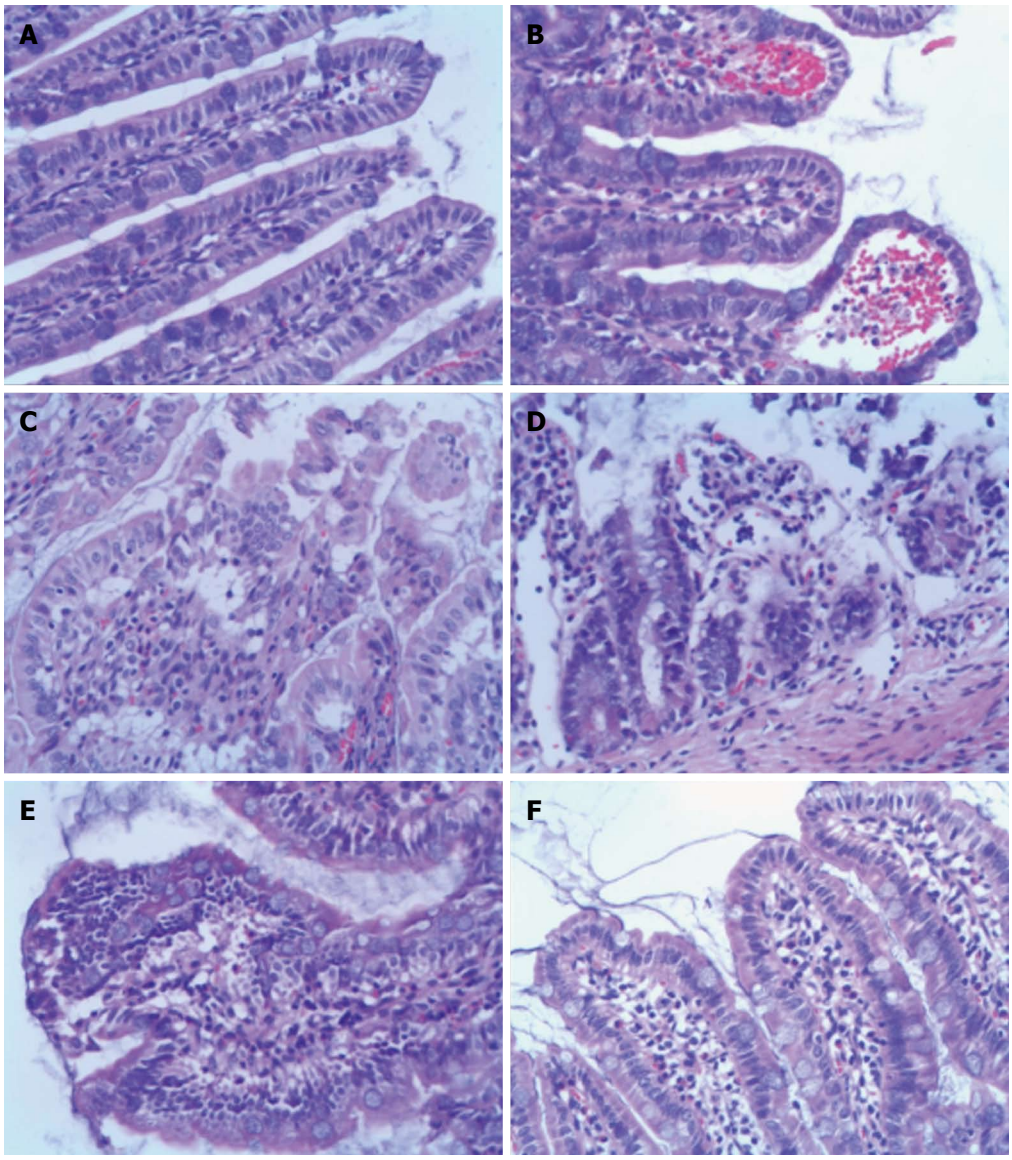

Figure 3 Histopathology of ileum sections at different time points after intestinal ischemia/reperfusion injury (hematoxylin and eosin, × 200). A: Sham group; the intestine showed normal villous architecture and glands, with no vascular congestion; B: In the ischemia/reperfusion (I/R) injury 2 h group, the degree of intestinal mucosa injury was marked with massive epithelial lifting down the sides of the villi and ulceration at the villous tips; C: At 6 h, there was intestinal mucosa degradation and disintegration of the lamina propria, hemorrhage, and ulceration; D: At 24 h, the damaged mucosa showed denuded villi with dilated capillaries and increased cellularity of the lamina propria; E: In the I/R injury group at 72 h, there was massive epithelial lifting down the sides of the villi and ulceration at the villous tips; F: However, at 144 h, the damaged mucosa had recovered.

1A and 1B), ability to differentiate hepatocytes *in vitro* (data not shown), and flow cytometry results (Figure 2). Most of the third-passage adherent cells were positive for CD90, CD29 and RT1A, and negative for the MSC markers, CD45, CD34 and RT1B. Furthermore, over the first three passages, the percentage of $CD90^+$ and $CD45^-$ cells rapidly increased from 80% to > 98% (Figure 2), which was in agreement with a previous study[26].

Confirmation of donor-derived BM MSCs

Labeled BM MSCs homing to the intestine were visible 2, 6, 24, 72 and 144 h after transplantation (Figure 1C). After the intestine was washed repeatedly with PBS, the labeled BM MSCs were still visible (Figure 1D), which indicated that the transplanted BM MSCs could home to the intestine and survive long term.

Histopathological examination

The histopathological findings showed intact villi with no epithelial disruption in the Sham groups. In the NS + I/R injury group, massive destruction of the villi and inflammatory cell infiltration into the lamina propria were evident. In contrast, intestinal samples in the BM MSCs + I/R injury group (BM MSCs group) had significantly less damage in the small intestine. Major pathological changes observed were slight hyperemia, edema, and inflammatory cell infiltration in the mucosa and submucosa, with most of the intestinal villi intact (Figures 3 and 4). Chiu's grade scores of the three groups are shown in Table 1.

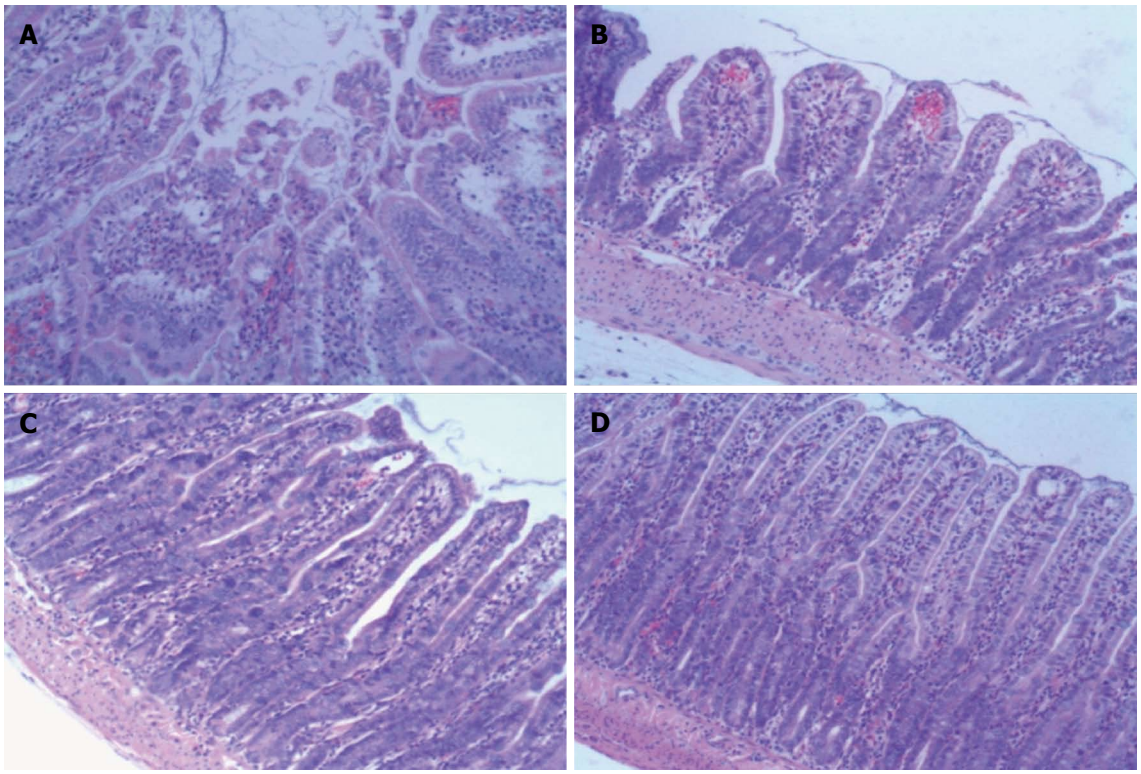

Figure 4 Histopathology of ileum sections of different groups at 6 h after ischemia/reperfusion injury (hematoxylin and eosin, × 100). A: In the ischemia/reperfusion (I/R) injury group there was marked intestinal mucosa injury at 6 h, with intestinal mucosa degradation and disintegration of the lamina propria, hemorrhage, and ulceration. B: In the bone-marrow mesenchymal stem cells + I/R injury group at 6 h, the damaged mucosa had recovered and there was extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria, massive epithelial lifting down the sides of the villi, and ulceration at the villous tips. C and D: In the anti-tumor necrosis factor (TNF)-α + I/R injury group and the anti-TNF-αR1-IgG + I/R injury group at 6 h, the damaged mucosa had almost recovered to resemble that in the Sham control group.

Table 1 Grade of intestinal mucosal injury after intestinal ischemia/reperfusion in the different groups

Group	Chiu's score				
	2 h	6 h	24 h	72 h	144 h
Sham group	0.5 ± 0.8	0.5 ± 0.5	0.7 ± 0.8	0.3 ± 0.8	0.7 ± 0.8
NS + I/R injury	18.0 ± 3.9[b]	27.8 ± 2.3[b]	23.7 ± 5.2[b]	19.0 ± 3.8[b]	11.0 ± 3.0[b]
BM MSCs + I/R injury	13.7 ± 3.3[b,d]	15.2 ± 2.9[b,d]	13.7 ± 1.5[b,d]	8.3 ± 2.3[b,d]	5.8 ± 2.6[b,d]
Anti-TNF-α + I/R injury		6.5 ± 1.2[d,f]			
Anti-TNF-αR1-IgG + I/R injury		7.7 ± 1.2[d,f]			

All values are mean ± SD (n = 6, three paraffin sections were prepared from each tissue sample. Two pathologists who were blinded to the source of the slides analyzed each slide); [b]$P < 0.01$ *vs* the Sham group, [d]$P < 0.01$ *vs* the saline (NS) + ischemia/reperfusion (I/R) injury group, [f]$P < 0.01$ *vs* bone-marrow mesenchymal stem cells (BM MSCs) + I/R injury group. TNF-α: Tumor necrosis factor-α.

Serum D-lactate and DAO

The levels of D-lactate and DAO significantly increased, reaching a peak at 6 h in the NS + I/R injury and BM MSCs + I/R injury groups, compared to the Sham group. This confirmed that I/R injury increased intestinal permeability.

The serum D-lactate and DAO levels in the NS + I/R injury group increased more than twofold compared to the Sham group at 2, 6 and 24 h after I/R ($P < 0.01$). However, the serum DAO levels in the BM MSCs + I/R injury group were significantly lower than in the NS + I/R group at 2, 6 and 24 h, and the serum D-lactate levels in the BM MSCs + I/R injury group were significantly lower than in the NS + I/R group at 6 and 24 h. At 6 h, the serum D-lactate and DAO levels in the anti-TNF-α + I/R injury and anti-TNF-αR-IgG + I/R injury groups were lower than in the NS + I/R injury group ($P < 0.01$; Table 2). At 72 and 144 h, the serum DAO levels in the NS + I/R and BM MSCs + I/R injury groups had reduced, but remained higher than in the Sham group, whereas D-lactate levels were not significantly different in the NS + I/R, BM MSCs + I/R and Sham groups at 144 h.

These data indicate that serum DAO is a more sensitive marker of intestinal permeability than D-lactate, and also that the administration BM MSCs or TNF-α block-

Table 2 Serum levels of diamine oxidase and D-lactate in a rat model of ischemia/reperfusion injury

Group	DAO (IU/mL)					D-lactate (μg/mL)				
	2 h	6 h	24 h	72 h	144 h	2 h	6 h	24 h	72 h	144 h
Sham group	2.08 ± 0.16	2.08 ± 0.75	2.03 ± 0.46	1.95 ± 0.36	2.24 ± 0.62	5.11 ± 0.24	5.30 ± 0.0.44	5.38 ± 0.45	5.46 ± 0.42	5.54 ± 0.69
NS + I/R injury	11.04 ± 0.59[b]	14.58 ± 2.01[b]	7.36 ± 1.28[b]	5.12 ± 0.66[b]	3.91 ± 0.59[b]	14.73 ± 1.37[b]	17.85 ± 1.86[b]	12.73 ± 0.56[b]	8.22 ± 1.78	6.54 ± 1.04
BM MSCs + I/R injury	8.16 ± 0.71[b,d]	11.36 ± 1.89[b,d]	5.04 ± 1.04[b,d]	4.93 ± 0.69[b]	3.55 ± 0.59[a]	12.62 ± 2.24[b]	13.40 ± 1.53[a,b]	9.80 ± 1.20b[d]	6.82 ± 0.80[b]	6.44 ± 0.83
Anti-TNF-α + I/R injury	-	7.99 ± 1.70[d]	-	-	-	-	12.77 ± 1.44[d]	-	-	-
Anti-TNF-αR1-IgG + I/R injury	-	7.83 ± 1.28[d]	-	-	-	-	12.16 ± 1.47[d]	-	-	-

All values are mean ± SD (n = 6). [a]P < 0.05, [b]P < 0.01 *vs* Sham group; [d]P < 0.01 *vs* the saline (NS) + ischemia/reperfusion (I/R) injury group.

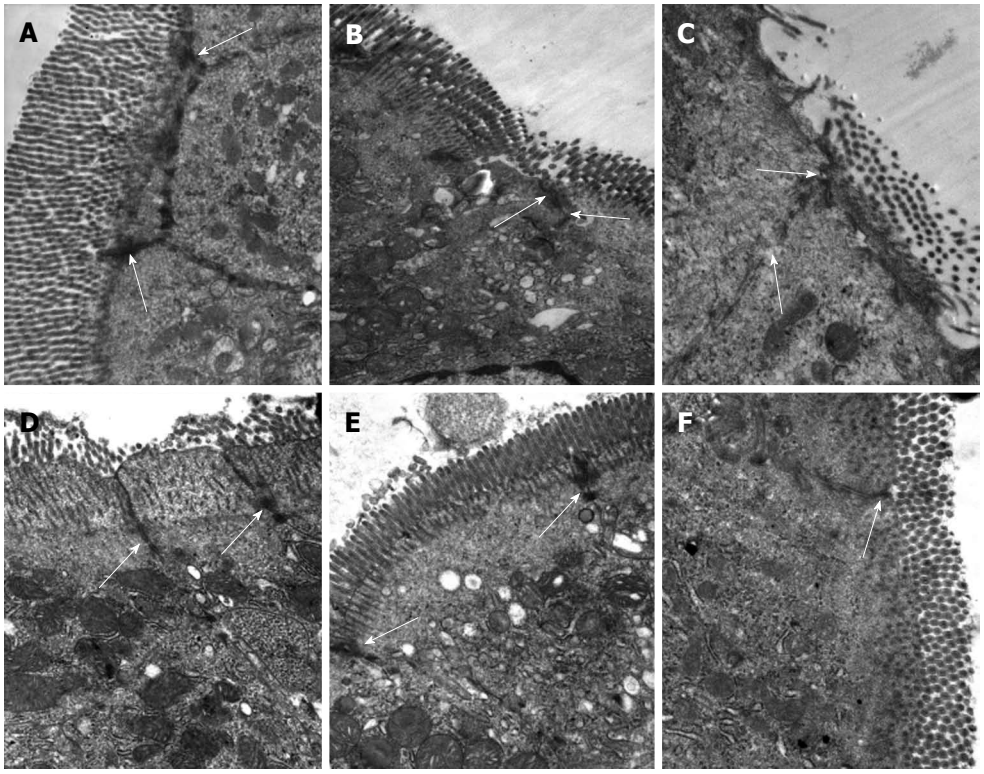

Figure 5 Bone-marrow mesenchymal stem cells and tumor necrosis factor-α blockade prevent ultrastructural pathological damage after intestinal ischemia/reperfusion injury. Transmission electron microscopy of the rat intestine after ischemia/reperfusion (I/R) injury. A: Epithelial cells and tight junctions (TJs) (arrows) were intact in the Sham group, × 30000; B: At 2 h after I/R injury, epithelial cells were swollen and shrunken, microvilli and organelles were normal, and TJs (arrows) were disrupted in the saline (NS) + I/R injury group, × 25000; C: At 6 h after I/R injury in the NS + I/R injury group, some microvilli were loose, TJs (arrows) were disrupted, and organelles were swollen with reduced electron density, × 30000; D: At 6 h after I/R injury and administration of bone-marrow mesenchymal stem cells, the microvilli and mitochondria of the endothelial cells were almost normal and TJs (arrows) were not disrupted, × 30000; E and F: TJs (arrows) between endothelial cells were intact 6 h after I/R injury in rats that received anti-tumor necrosis factor (TNF)-α IgG + I/R antibody (E, × 25000) or anti-TNF-α R1 antibody; (F, × 30000) before I/R injury.

ade reduced the permeability of the small intestine and accelerated the recovery of intestinal barrier function after I/R injury in rats.

Ultrastructural characteristics of the intestinal mucosa

Compared to the Sham group, we observed obvious ultrastructural changes in the intestinal mucosa after I/R injury in the rats from the NS + I/R group. Epithelial cell microvilli were sparsely distributed, disarranged and distorted, and the epithelial cells were swollen or shrunken. The mitochondrial matrices were swollen, cristae were broken, and numerous TJs were disrupted. There was no disruption of TJs in the BM MSCs + I/R injury group, and only swelling of the epithelial cells was observed. The ultrastructural pathological changes in the groups treated with anti-TNF-α and anti-TNF-αR-IgG were also less severe than in the NS + I/R injury group (Figure 5).

Expression of ZO-1 protein

Immunohistochemical analysis revealed strong ZO-1 expression in the intestinal tissue of the Sham group. In the intestinal tissue of the NS + I/R injury group, ZO-1 was expressed at low levels 2 h after injury, slightly in-

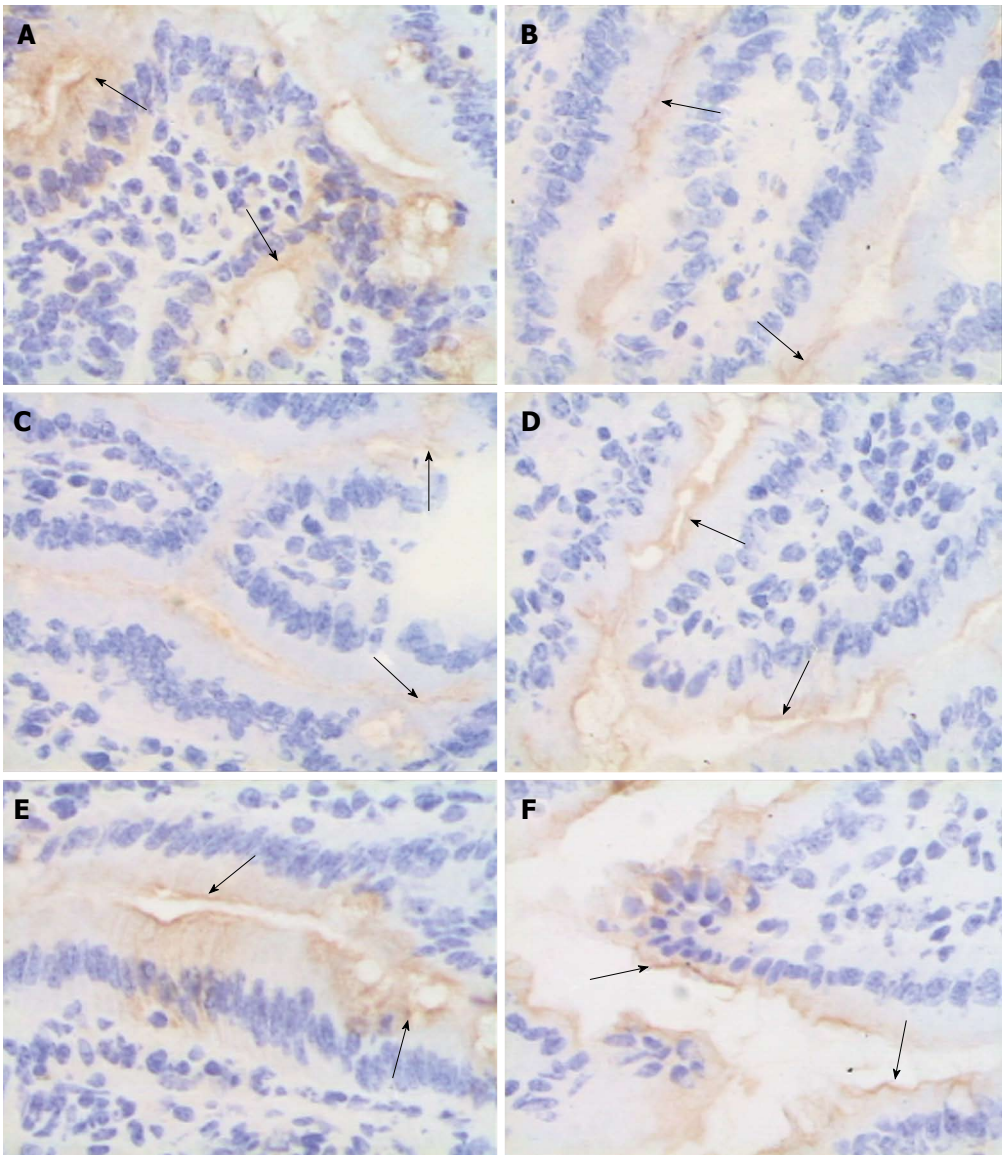

Figure 6 Bone-marrow mesenchymal stem cells and tumor necrosis factor-α blockade attenuate zona occludens 1 downregulation after intestinal ischemia/reperfusion injury. The mucosal tissue sections after ischemia/reperfusion (I/R) injury were immunohistochemically labeled for zona occludens 1 (ZO-1) (brown) and counterstained with hematoxylin (blue). A: Sham group; B and C: In the normal saline + I/R injury group, decreased ZO-1 staining (arrows) was observed in the epithelial cells 2 h (B) and 6 h (C) after I/R; D-F: ZO-1 was not obviously affected in the bone-marrow mesenchymal stem cells + I/R injury group (D), anti-tumor necrosis factor (TNF)-α + I/R injury group (E), or anti-TNF-α R1 antibody + I/R injury group (F) at 6 h; original magnification, × 400.

creased at 6 and 24 h, and by 72 h, ZO-1-positive signals were detected throughout the entire intestine (Figure 6). Western blot analysis confirmed that ZO-1 expression decreased more significantly in the NS + I/R group than the BM MSCs + I/R injury group, particularly at 6 h (Figure 7). Consistent with the immunohistochemical results, western blotting indicated that ZO-1 expression was significantly higher in the BM MSCs + I/R injury group and the two antibody-treated groups at 6 h than in the NS + I/R injury group (Figure 8).

Effect of I/R injury on serum TNF-α levels

Compared to the Sham group, the serum TNF-α levels increased significantly, peaking at 6 h, in the NS + I/R injury group. The serum level of TNF-α was significantly lower in the BM MSCs+ I/R injury group at 6 and 24 h than the NS + I/R injury group ($P < 0.05$, Table 3). The morphological abnormalities after intestinal I/R injury were positively correlated with serum TNF-α levels (Table 4).

DISCUSSION

I/R injury to the gut is a common event in a variety of clinical conditions, such as trauma, burn injuries, septic shock, heart and aortic surgery, and liver and small bowel transplantation[27,28]. Intestinal I/R results in edema, apoptosis, necrosis of epithelial cells, disruption of mucosal integrity and small intestine function, which in turn increases mucosal and vascular permeability, bacte-

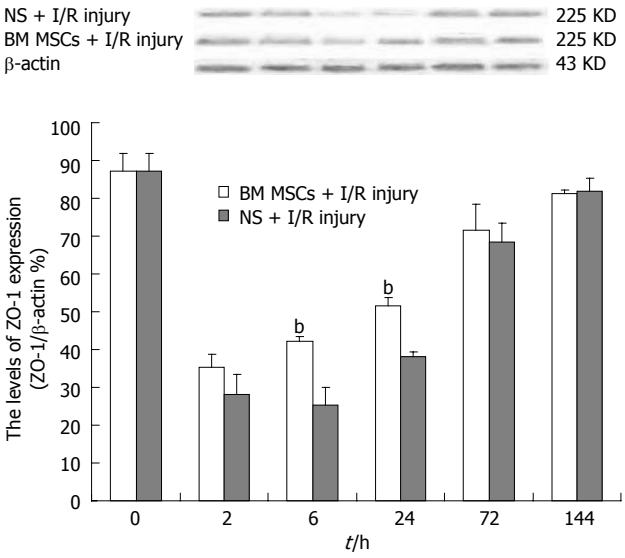

Figure 7 Bone-marrow mesenchymal stem cells attenuate zona occludens 1 downregulation after intestinal ischemia/reperfusion injury. Representative western blots and quantification of zona occludens 1 (ZO-1) protein expression in the intestinal mucosa. ZO-1 expression was significantly lower in the saline (NS) + ischemia/reperfusion (I/R) injury group than the bone-marrow mesenchymal stem cells (BM MSCs) + I/R injury group at 6 h (25.35 ± 4.58% *vs* 42.32 ± 1.26%; $P < 0.01$). Actin was used as a loading control. Values are shown as the mean ± SD ($n = 3$ rats per group); $^{b}P < 0.01$ BM MSCs +I/R injury group *vs* the NS + I/R injury group [one-way ANOVA followed by the least significant difference (LSD) test].

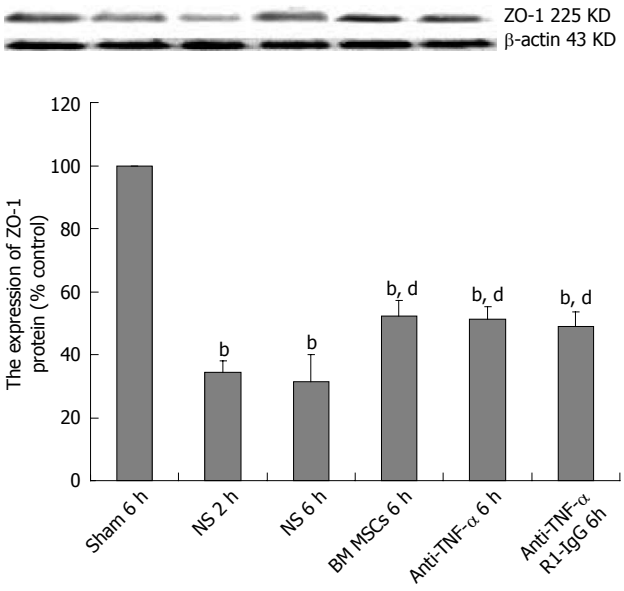

Figure 8 Bone-marrow mesenchymal stem cells and tumor necrosis factor-α blockade attenuate zona occludens 1 downregulation after intestinal ischemia/reperfusion injury. Representative western blots and quantification of zona occludens 1 (ZO-1) protein expression in the intestinal mucosa. Actin was used as a loading control. Values are the mean ± SD ($n = 3$ rats per group); $^{b}P < 0.01$ *vs* Sham group; $^{d}P < 0.01$ *vs* the saline (NS) + ischemia/reperfusion (I/R) injury group (one-way analysis of variance followed by the LSD test). BM MSCs: Bone-marrow mesenchymal stem cells; Anti-TNF: Anti-tumor necrosis factor.

rial translocation, as well as the risk of systemic inflammation response syndrome, multiple organ dysfunction and death[29,30]. Until recently, no effective treatments existed for intestinal I/R injury. Research has suggested that BM MSCs could possibly play a role in the treatment of I/R injury in the heart, kidney and brain[31-33]; however, studies of the effects of BM MSCs in intestinal disorders are scarce. In the current study, the therapeutic potential of BM MSCs was evaluated in an experimental rat model of I/R injury, which led to disruption of intestinal mechanical barrier function. The results of this study suggest that BM MSCs can effectively reduce both the intestinal permeability and pathological damage associated with I/R injury. BM MSCs have the potential for multidirectional differentiation. They participate in colonic mucosal regeneration[34]. In this study, intestinal I/R injury lead to necrosis and the loss of a large number of intestinal epithelial cells. BM MSCs could reduce I/R injury and protect the intestine.

Stem cell homing processes are thought to play a crucial role in the success of cell therapy for organ function disorders. Intravenous or intra-arterial infusions of BM MSCs often result in the entrapment of the administered cells in organ capillary beds, especially in the lung and the liver[35]. The transplantation of BM MSCs by intravenous or intra-arterial routes usually results in a low engraftment rate; therefore, increasing the number of MSCs within the injured area would improve the efficacy of cell therapy. Zhang *et al*[36] used gene-modified MSCs to enhance the homing rate of BM MSCs to the irradiated intestine by 20% using an intravenous delivery route. However, using viral vectors to transfect MSCs may decrease the viability of MSCs. In this study, we directly injected MSCs into the wall of the intestine after I/R injury, which significantly increased the homing of MSCs into the I/R damaged intestinal mucosa. This indicated that the direct injection of BM MSCs into the intestine may provide a better method to enhance the homing rate.

The intestinal mucosal barrier is composed of mucosal fluid, microvilli, epithelial mucosal cell TJs, and other special structures. TJs are the most important structures in the mucosal barrier. The mechanisms responsible for intestinal I/R injury include cytotoxic effects and alterations in the structure of the intestinal mucosa[15]. However, few studies have examined the intestinal mucosa and TJ ultrastructure during I/R injury, and the role and mechanism of action of BM MSCs in intestinal I/R injury are unclear. In the present study, we found that severe intestinal mucosal damage occurred 2, 6 and 24 h after I/R injury. The morphological alterations to the intestinal mucosa included the shedding of epithelial cells, fracturing of villi, fusion of adjacent villi, mucosal atrophy and edema. Disruption of TJs between enterocytes, and damage to the mitochondria and endoplasm were also observed. Although damage to the intestinal mucosa plays a significant role in the permeability of the intestine, the mechanisms which cause this damage are poorly characterized. Moreover, we observed that the intestinal permeability increased 2, 6 and 24 h after I/R

Table 3 Serum levels of tumor necrosis factor-α in a rat model of ischemia/reperfusion injury

Group	Tumor necrosis factor-α (pg/mL)				
	2 h	6 h	24 h	72 h	144 h
Sham group	87.07 ± 6.47	84.45 ± 4.18	87.60 ± 6.25	83.91 ± 6.67	86.54 ± 5.62
NS + I/R injury	226.32 ± 11.94[b]	332.95 ± 49.03[d]	221.80 ± 16.06[d]	180.87 ± 8.63[b]	134.84 ± 7.18[b]
BM MSCs + I/R injury	214.21 ± 17.77[b]	236.76 ± 20.66[b,d]	190.39 ± 4.24[a,d]	177.00 ± 2.52[b]	91.67 ± 3.84[b]

All values are mean ± SD (n = 6). [b]P < 0.01 *vs* Sham group; [d]P < 0.01 *vs* the saline (NS) + ischemia/reperfusion (I/R) injury group. BM MSCs: Bone-marrow mesenchymal stem cells.

Table 4 Serum levels of tumor necrosis factor-α and intestinal mucosal injury grade after intestinal ischemia/reperfusion

Group	TNF-α (pg/mL)					Chiu's grade scores				
	2 h	6 h	24 h	72 h	144 h	2 h	6 h	24 h	72 h	144 h
Saline + I/R injury group	243.27	290.41	210.51	186.58	132.55	19	28	18	14	10
	212.21	286.35	241.42	189.44	125.39	14	27	21	14	8
	221.76	283.66	203.19	174.82	137.45	17	30	17	13	10
	235.42	295.87	210.37	175.76	129.57	18	28	19	13	9
	229.54	345.78	226.96	189.26	145.57	18	29	20	14	12
	215.74	395.61	238.32	169.34	138.51	15	30	21	12	10
Spearman						0.947	0.931	0.961	0.971	0.956
P						0.004	0.011	0.002	0.001	0.003

The degree of relationship between tumor necrosis factor (TNF)-α and the Chiu risk score was evaluated by bivariate correlation. The results were statistically significant when P < 0.05, and was highly significant when P < 0.01. I/R: Ischemia/reperfusion.

injury, with simultaneous disruption in TJ integrity. Additionally, the administration of BM MSCs significantly attenuated the histological damage due to I/R injury (Figure 4) and reduced intestinal permeability (Table 2), compared with the NS + I/R injury group. Therefore, we hypothesized that changes in intestinal permeability may occur due to the disruption of TJs between intestinal mucosa epithelial cells.

To understand the mechanism of TJ disruption, we investigated the expression of ZO-1. ZO-1 was the first TJ-related protein to be identified[37], and it connects the actin cytoskeleton to the transmembrane occludin proteins[38]. ZO-1 plays a vital role in the maintenance of intestinal mucosal barrier integrity and TJs during pathological insults[39]. In this study, ZO-1 expression in the intestinal mucosa significantly decreased after I/R injury; thus, we concluded that decreased ZO-1 expression lead to TJ disruption and possibly increased gut permeability.

Next, we examined the mechanism of TJ disruption and reduced ZO-1 protein expression during I/R injury. We observed that TNF-α increased at 2, 6 and 24 h after I/R injury, and correlated with ZO-1 downregulation and TJ disruption. The pathophysiological processes of I/R injury *in vivo* are complex, and it is thought that TNF-α may play an important role. Inflammation involves the sequential activation of signaling pathways which result in the production of pro- and anti-inflammatory mediators during I/R injury. Amongst the proinflammatory mediators, the TNF-α and TNF-αR1 systems play central roles in the physiological regulation of intestinal barrier function[40,41], and both TNF-α and interferon (IFN)-γ can induce intestinal epithelial barrier dysfunction[42]. Some cytokines can induce endocytosis[43] and internalization of epithelial TJ proteins[44]. In mice with fulminant hepatic failure, reduced expression of occludin in intestinal epithelial cells was linked to increased TNF-α production[4]. TNF-α can also induce an increase in Caco-2 cell TJ permeability *via* nuclear factor-κB activation, leading to downregulation of ZO-1 protein expression and altered junctional localization[38,45]. We hypothesize that TNF-α acts as an initiator, which can induce expression of other cytokines such as IL-6 and IFN-γ, which then initiate and aggravate the development of I/R injury, and disrupt intestinal TJs.

After the transplantation of BM MSCs, the serum TNF-α level significantly decreased, the damaged mucosa recovered, ZO-1 expression increased and intestinal permeability significantly improved. TNF-α is known to inhibit the expression of ZO-1[44], and if the TJs are damaged, intestinal barrier dysfunction will occur. Research has confirmed that BM MSCs can inhibit the generation of TNF-α in dendritic cells *in vitro*[46,47], and therefore we hypothesized that BM MSCs could repair intestinal I/R injury by inhibiting the release of TNF-α. In order to further study the role of TNF-α, we used anti-TNF-α and anti-TNFR antibodies. The TNF-α antibody neutralizes TNF-α, whereas the anti-TNFR antibody blocks the binding of TNF-α to the TNF-α receptor. TNF-α blockade significantly decreased the severity of I/R injury, which indicates that TNF-α is an important mediator of intestinal mucosa damage during I/R injury. These findings suggest that I/R injury increases TNF-α, leading to downregulation of ZO-1 protein expression, whereas BM MSCs can inhibit pro-

duction of TNF-α, leading to increased expression of ZO-1 and reduced intestinal mucosa damage. These effects were observed over a relatively short observation period, and long-term studies are required to elucidate if TNF-α exerts long-lasting effects during I/R injury.

In summary, this study demonstrates that the submucosal infusion of BM MSCs decreased intestinal permeability and preserved intestinal mechanical barrier function after I/R injury in rats, in a mechanism linked to reduced serum TNF-α levels and the increased expression of the intestinal TJ protein ZO-1. Future studies using exogenous or autologous BM MSCs to prevent or modulate intestinal I/R injuries are required to assess the clinical potential of BM MSCs. The mechanisms by which BM MSCs and TNF-α blockade protect against I/R-induced disruption of intestinal barrier function remain to be further investigated.

Disruption of the intestinal mucosa and the consequent increase in permeability after I/R injury may be due to reduced levels of the TJ-associated protein, ZO-1. BM MSCs restored the epithelial structure, promoted the recovery of intestinal permeability, increased ZO-1 protein expression and protected against intestinal I/R injury. TNF-α plays an important role in the ability of BM MSCs to protect against intestinal I/R injury, as the epithelial structure remained normal, and changes in intestinal permeability and ZO-1 protein expression were reduced when rats were treated with anti-TNF-α IgG antibody or anti-TNF-α R1 antibodies before I/R injury. This study confirms that high levels of TNF-α damage TJs and downregulate ZO-1 protein expression *in vivo*. The mechanism of TNF-α-induced change during I/R injury is complex and requires further study.

COMMENTS

Background

Digestive organ transplantation and other abdominal surgical procedures can result in different degrees of intestinal ischemia/reperfusion (I/R) injury, which can delay patient recovery and lead to systemic organ failure. Therefore, intestinal I/R injury is an important clinical issue. Bone-marrow mesenchymal stem cells (BM MSCs) can protect against I/R injury; however, the mechanism is unclear. Although previous studies have provided an insight into the molecular structure of tight junctions (TJs), much less is known about TJ functionality under physiological or pathophysiological conditions. Few studies have described the intestinal mucosa ultrastructure or changes in TJs during intestinal I/R injury. In this study, the authors used a rat model of intestinal I/R injury to investigate the effect of BM MSCs on the intestinal mucosa ultrastructure, with an emphasis on the mechanisms of intestinal barrier dysfunction.

Research frontiers

In this study, the authors demonstrated that the submucosal infusion of BM MSCs decreased intestinal permeability and preserved intestinal mechanical barrier function after I/R injury in rats, in a mechanism linked to reduced serum tumor necrosis factor (TNF)-α levels and the increased expression of the intestinal TJ protein zona occludens (ZO)-1. Altered serum TNF-α levels play an important role in the ability of BM MSCs to protect against intestinal I/R injury.

Innovations and breakthroughs

Recent reports have highlighted the importance of BM MSCs reducing intestinal I/R injury in rats. Although previous studies have provided an insight into the molecular structure of TJs, much less is known about TJ functionality under physiological or pathophysiological conditions. Few studies have described the intestinal mucosa ultrastructure or changes in TJs during intestinal I/R injury. This is believed to be the first study to report that BM MSCs reduce rat intestinal I/R injury, ZO-1 downregulation, and TJ disruption *via* a TNF-α-regulated mechanism.

Applications

By understanding how BM MSCs reduce rat intestinal I/R injury, this study may represent a future strategy for therapeutic intervention in the treatment of patients with digestive organ transplantation and other abdominal surgical procedures that result in different degrees of intestinal I/R injury, which can delay patient recovery and lead to systemic organ failure.

Terminology

TJs are the main structures responsible for restricting the paracellular movement of compounds across the intestinal mucosa. Structurally, TJs are composed of cytoplasmic proteins, including ZO-1-3 and two distinct transmembrane proteins, occludin and claudin, which are linked to the actin-based cytoskeleton. ZO-1, as a scaffold for the organization of transmembrane TJ proteins, also recruits various signaling molecules and the actin cytoskeleton to TJs.

Peer review

This paper shows the impact of BM MSCs on rat intestinal I/R injury. This study will be of interest and the paper is clearly written.

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BRIEF ARTICLE

Risk factors for colonoscopic perforation: A population-based study of 80118 cases

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Abstract

AIM: To assess the incidence and risk factors associated with colonic perforation due to colonoscopy.

METHODS: This was a retrospective cross-sectional study. Patients were retrospectively eligible for inclusion if they were 18 years and older and had an inpatient or outpatient colonoscopy procedure code in any facility within the Geisinger Health System during the period from January 1, 2002 to August 25, 2010. Data are presented as median and inter-quartile range, for continuous variables, and as frequency and percentage for categorical variables. Baseline comparisons across those with and without a perforation were made using the two-sample *t*-test and Pearson's χ^2 test, as appropriate.

RESULTS: A total of 50 perforations were diagnosed out of 80118 colonoscopies, which corresponded to an incidence of 0.06% (95%CI: 0.05-0.08) or a rate of 6.2 per 10000 colonoscopies. All possible risk factors associated with colonic perforation with a *P*-value < 0.1 were checked for inclusion in a multivariable log-binomial regression model predicting 7-d colonic perforation. The final model resulted in the following risk factors which were significantly associated with risk of colonic perforation: age, gender, body mass index, albumin level, intensive care unit (ICU) patients, inpatient setting, and abdominal pain and Crohn's disease as indications for colonoscopy.

CONCLUSION: The cumulative 7 d incidence of colonic perforation in this cohort was 0.06%. Advanced age and female gender were significantly more likely to have perforation. Increasing albumin and BMI resulted in decreased risk of colonic perforation. Having a colonoscopy indication of abdominal pain or Crohn's disease resulted in a higher risk of colonic perforation. Colonoscopies performed in inpatients and particularly the ICU setting had substantially greater odds of perforation. Biopsy and polypectomy did not increase the risk of perforation and only three perforations occurred with screening colonoscopy.

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Key words: Colonoscopic perforation; Colon cancer; Endosocopy

Core tip: This study is unique because we have used state of the art electronic medical records to collect information about risk factors which can predispose patient to a high risk of perforation. We have looked into multiple risk factors including but not limited to serum albumin, serum creatinine, body mass index (BMI), inpatient and outpatient colonoscopy and intensive care unit (ICU) patients. Limited literature is available about the above mentioned risk factors and there propensity

to cause perforation. The important findings deduced from this research can have important implication in day to day practice of colonoscopy. The findings of Albumin, BMI, and Inpatient and out patient colonoscopy particularly performing colonoscopy in ICU setting predisposing to higher risk of perforation are crucial piece of information that can help physician in considering available alternatives which in turn may help to reduce the number of colonoscopic perforations.

Hamdani U, Naeem R, Haider F, Bansal P, Komar M, Diehl DL, Kirchner HL. Risk factors for colonoscopic perforation: A population-based study of 80118 cases. *World J Gastroenterol* 2013; 19(23): 3596-3601 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3596.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3596

INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of cancer-related death in the United States[1]. Early detection benefits patients and increases their quality of life, but also reduces health care expenditures. The ability of colonoscopy to detect polyps and colorectal cancer has been shown to reduce mortality and morbidity associated with this cancer[2,3]. In July 2001, Medicare began covering screening colonoscopy for individuals over the age of 50 at average risk for colorectal cancer and depending on the detection of polyps, at variable intervals thereafter. Since that time, the use of colonoscopy has been increasing[4-6].

Colonoscopy is generally regarded as a safe procedure; potential complications include perforation, post-polypectomy bleeding and post-polypectomy syndrome[7]. The incidence of colonic perforation ranges from 0.005% and 0.63% with the majority of patients requiring laparotomy for repair[8-12]. Colonoscopic perforation occurs due to one of three mechanisms; mechanical forces from the endoscope, barotrauma from air insufflation, or as a direct result of a therapeutic procedure (*e.g.*, polypectomy). To better understand the risk factors associated with colonoscopic perforation, we conducted a large cross-sectional study to estimate the incidence of this serious complication, and to examine potential contributing effects of demographic and medical characteristics of patients.

MATERIALS AND METHODS

Study cohort, design, and setting

This was a retrospective cross-sectional study. Patients were retrospectively eligible for inclusion if they were 18 years and older and had an inpatient or outpatient colonoscopy procedure code in any facility within the Geisinger Health System (GHS) during the period from January 1, 2002 to August 25, 2010. GHS is a primary care and multispecialty medical practice located in central and northeast Pennsylvania and is the largest rural not-for-profit health system in the nation. GHS uses health information technology infrastructure for managing and using patient data. Colonoscopy procedures were identified by the presence of current procedural terminology (CPT) 2005 codes 45378-45387, 45391, and 45392.

The study outcome was the diagnosis of colonic perforation using International Classification of Disease, 9th revision (ICD-9) codes 569.83 and 998.2, defined as perforation of intestine and accidental puncture or laceration during a procedure, 7 d after the day of colonoscopy. We specifically looked at the 7 d post colonoscopy for perforation since previous studies have shown that almost all post-colonoscopy perforations were detected within this time frame[13,14]. Both inpatient and outpatient procedures were included.

Variables obtained from the electronic health record included age at colonoscopy, gender, body mass index (BMI), albumin, serum creatinine, operator specialty (surgeon or gastroenterologist), and indications for the colonoscopy (identified by procedure codes in the colonoscopy report). Race was not assessed for analysis since the primary care population seen in Geisinger Health System is > 95% Caucasian.

Data on comorbid health conditions were also collected including history of coronary artery disease, congestive heart failure, peripheral arterial disease, cerebrovascular disease, dementia, chronic obstructive pulmonary disease, connective tissue disease, peptic ulcer disease, liver disease, diabetes mellitus, hemiplegia, chronic kidney disease, leukemia, lymphoma, metastatic cancer, and acquired immune deficiency syndrome.

Statistical analysis

Data are presented as median and inter-quartile range, for continuous variables, and as frequency and percentage for categorical variables. Baseline comparisons across those with and without a perforation were made using the two-sample *t*-test and Pearson's χ^2 test, as appropriate. The incidence of 7-d post-colonoscopy perforations was calculated as the number of colonic perforations divided by the total number of colonoscopies, and expressed as both percentage and as an incidence rate (*e.g.,* number of perforations per 10000 colonoscopies). The count of comorbid conditions was summarized as a general indicator of health.

The log-binomial model was used to estimate the incident rate ratios (IRR) for risk factors found to vary across the two groups. A fully adjusted model was then developed to identify those risk factors predictive of perforation. Variables were considered for inclusion in the model if they were found to vary between groups at a significance level of $P < 0.10$. Backward elimination was performed to obtain a final model that retained clinically meaningful predictors. Results are presented as RR and corresponding 95%CI. The analysis was performed using SAS v9.2 (SAS Institute, Inc., Cary, NC, United States) and R v2.13 (R Development Core Team, www.r-project-org)[15].

Table 1 Potential risk factors stratified by colonic perforation *n* (%)

	No perforation (*n* = 80068)	Perforation (*n* = 50)	*P*-value
Age category (yr)			< 0.0001
18-50	13698 (17.11)	5 (10.00)	
50-65	38695 (48.33)	10 (20.00)	
65-80	22954 (28.67)	20 (40.00)	
80+	4271 (5.90)	15 (30.00)	
Gender	*n* = 80059	*n* = 50	0.0183
Male	38972 (46.68)	16 (32.00)	
Female	41087 (51.32)	34 (68.00)	
BMI (median, IQR)	28.66 (25.14, 32.92)	26.27 (20.70, 28.55)	0.0002
(5.35% unknown)[3]	*n* = 79615	*n* = 48	
Operator specialty[4]	*n* = 78421	*n* = 46	0.2290
Surgery	13826 (17.63)	5 (10.87)	
Gastroenterology	64595 (82.37)	41 (89.13)	
Type of colonoscopy[2]			< 0.0001
Therapeutic	37867 (47.29)	13 (26.53)	
Polypectomy	16367 (20.44)	6 (12.42)	
Dilation	97 (0.12)	2 (4.08)	
Biopsy	18807 (23.49)	2 (4.08)	
Other[1]	2596 (3.2)	3 (6.1)	
Screening	29898 (37.34)	3 (6.12)	
Diagnostic	12303 (15.37)	33 (67.35)	
Number of Interventions performed[5]			0.0342
1	73919 (92.32)	45 (90.00)	
2	5527 (6.90)	3 (6.00)	
3	622 (0.78)	2 (4.00)	
Count of morbidities			0.2004
0	55601 (69.4)	31 (62.0)	
1	17330 (21.6)	11 (22.0)	
2+	7137 (8.9)	8 (16.0)	
Setting			< 0.0001
Inpatient	4132 (5.2)	23 (46.0)	
Outpatient	75936 (94.8)	27 (54.0)	
ICU	85 (0.1)	9 (18.0)	< 0.0001
Indications for CP			
Abdominal pain	3623 (4.52)	7 (14.00)	0.0070
Anemia	1875 (2.34)	5 (10.00)	0.0063
Bleeding	3615 (4.51)	7 (14.00)	0.0070
Crohn's disease	329 (0.41)	2 (4.00)	0.0183
Diarrhea	2565 (3.20)	0 (0)	0.4115
Diverticulosis of colon	15635 (19.53)	10 (20.00)	0.9328
Obstruction	416 (0.52)	0 (0)	0.9999
Ulcerative colitis	920 (1.15)	1 (2.00)	0.4391
Weight loss	464 (0.58)	1 (2.00)	0.2526
Creatinine (median, IQR) (12% unknown)	0.9 (0.7, 1.0)	0.9 (0.7, 1.1)	0.9824

[1]Includes foreign body removal, hemostasis; [2]Info to ascertain type of colonoscopy in one perforation not available; [3]Two body mass index (BMI) in perforation group not available; [4]Operator specialty of 4 perforations are other then gastroenterology and general surgery; [5]Number of interventions includes biopsy, polypectomy ,dilations and hemostasis. ICU: Intensive care unit; CP: Cerebral palsy; IQR: Interquartile range.

RESULTS

A total of 50 perforations were diagnosed out of 80118 colonoscopies, which corresponded to an incidence of 0.06% (95%CI: 0.05-0.08) or a rate of 6.2 per 10000 colonoscopies. Thirty-nine patients underwent emergent exploratory laparotomy and 11 were managed conservatively.

Patients that had a perforation within 7 d of their procedures were more likely to be older, female gender, lower BMI, have more comorbidities, and a lower albumin value (Table 1). Indications for colonoscopy including abdominal pain, anemia, and bleeding were reported more in those with a perforation as compared to the non-perforation group. Operator specialty and creatinine values were not found to vary between groups. The presence of end stage renal disease (ESRD) and prior abdominal surgeries also were not associated with colonic perforations in our cohort.

Based on the findings in Table 1, those risk factors meeting the criteria of a $P < 0.1$ were further described. Table 2 reports the number of perforations and the incidence rate per 10000 patients stratified by these important factors. For every year increase in age, the risk of a perforation increased by 7% (95%CI: 5-9) with the incidence of perforation increasing from 2.6 cases per 10000 in the 50-64 year old age group to 31.7 cases per 10000 in the 80+ year old age group. Females were twice as likely to have a perforation compared to males. Lower BMI resulted in a higher risk of perforation. Decreased albumin levels (≤ 4.0) (closest prior to colonoscopy) were associated with an increased risk of colonic perforation (IRR = 7.8, 95%CI: 4.1-14.6). There was also a significant difference of perforation rate between colonoscopy performed in an inpatient and outpatient setting. Inpatients were much more likely to have perforation compared with outpatients (55.4 and 3.6 cases per 10000, respectively). Similarly, the risk of perforation increased in intensive care unit (ICU)-patients compared to non-ICU patients.

All possible risk factors associated with colonic perforation with a *P*-value < 0.1 were checked for inclusion in a multivariable log-binomial regression model predicting 7-d colonic perforation. The final model resulted in the following risk factors which were significantly associated with risk of colonic perforation: age, gender, BMI, albumin level, ICU patients, inpatient setting, and abdominal pain and Crohn's disease as indications for colonoscopy. Approximately 21% of the patients did not have an albumin laboratory result available for analysis. Therefore, a model was fit with and without including albumin. Also, based on the descriptive results albumin was categorized at 4.0. The estimates from the final models are displayed in Table 3.

DISCUSSION

In reviewing literature from 1975 onward, we observed that the incidence rate of colonic perforation ranges between 0.005% to 0.63%. We noticed a gradual decline in the incidence of colonic perforation which has reached a plateau in the last 10 years. The differences in the incidence rates in this study compared to those in the literature can possibly be attributed to the way the studies were conducted. For example Sieg *et al*[16] and Rathgaber *et al*[17] both reported low incidence rate of perforation (0.005 and 0.01 respectively). Sieg *et al*[16] looked prospectively at 82, 16 colonoscopies but there was a selection

Table 2 Incidence of 7-d colonic perforation risk by important risk factors

Patient variable	Frequency	Perforations	Incidence per 10000	Incident rate ratio (95%CI)
Total	80118	50	6.2	-
Age (yr)				1.07[1] (1.05, 1.09)
18-49	13703	5	3.6	
50-64	38705	10	2.6	
65-79	22974	20	8.7	
80+	4736	15	31.7	
Gender				
Female	41121	34	8.3	-
Male	38988	16	4.1	0.50 (0.27, 0.90)
BMI (kg/m^2)				0.91[1] (0.86, 0.96)
< 24 (normal weight)	18019	21	11.7	
25-29 (overweight)	26873	17	6.3	
30+ (obese)	30873	10	3.2	
Type of Colonoscopy				
Therapeutic	37880	13	3.4	-
Screening	29901	3	1.0	0.29 (0.08, 1.03)
Diagnostic	12336	33	26.8	7.79 (4.10, 14.80)
Albumin result (percentile cut-offs)				0.15[1] (0.12, 0.20)
≤ 4.0	16537	36	21.8	7.76 (4.12, 14.64)
> 4.1	46366	13	2.8	-
ICU patients	94	9	957.4	186.9 (93.5, 373.5)
Non-ICU patients	80024	41	5.1	-
Inpatients	4155	23	55.4	15.6 (8.9, 27.1)
Outpatients	75963	27	3.6	-
Indications for CP				
Abdominal pain				3.4
Yes	3630	7	19.3	(1.5, ,7.6)
No	76488	43	5.6	-
Anemia				4.6
Yes	1880	5	26.6	(1.8, 11.6)
No	78238	45	5.8	-
Bleeding				3.4
Yes	3622	7	19.3	(1.5, 7.6)
No	76496	43	5.6	-
Crohn's disease				
Yes	331	2	60.4	10.0
No	79787	48	6	(2.5, 41.2)

[1]Variable was treated as continuous in the estimation of the incident rate ratio. ICU: Intensive care unit; CP: Cerebral palsy; BMI: Body mass index.

Table 3 Multivariate log-binomial regression results predicting 7-d post colonoscopic perforation

Risk factor	Model without albumin	Model with albumin
Age	1.04 (1.01, 1.06)	1.03 (1.01, 1.05)
BMI	0.96 (0.91, 1.00)	0.94 (0.90, 0.99)
ICU	9.37 (4.42, 19.88)	5.83 (2.80, 12.14)
Inpatient	18.08 (8.58, 38.17)	11.05 (5.14, 23.75)
Type of colonoscopy		
Therapeutic	-	-
Screening	0.25 (0.07, 0.87)	0.17 (0.04, 0.76)
Diagnostic	12.93 (6.65, 25.13)	15.33 (7.79, 30.18)
Abdominal pain	5.32 (2.40, 11.82)	5.79 (2.64, 12.74)
Crohn's disease	11.26 (3.88, 32.70)	5.16 (1.79, 14.88)
Albumin (≤ 4.0)	-	3.58 (1.72, 7.47)

BMI: Body mass index; ICU: Intensive care unit.

bias since only perforations that required surgical intervention were included in the study. Similarly, Rathgaber and Wick's study of 12407 colonoscopies, complications were gathered by monthly retrospective review of all hospitalizations and patient phone calls. Anderson *et al*[18] reported an incidence 0.19% in 10486 colonoscopies and Gatto *et al*[13] found 0.196% in 39286 colonoscopies. Both studies primarily looked at an older patient population which may have contributed to the higher rate of colonoscopic perforations.

This study looked at patients 18 years or older. By including a wider range of patients, the current findings are likely to be more representative of the true incidence of perforation. We found that age greater than 65 years was a significant predictor for risk of perforation. This finding is in congruence with other studies[12-14,19] that found increased age as an independent risk factor for perforation.

We found that the female gender is predisposed to a higher risk of perforation as compared to the male gender. Anderson *et al*[18] and Korman *et al*[12] also found female gender to be an independent risk factor for perforation. In contrast, Arora *et al*[19] did not find female gender as a significant risk factor for perforation in 277434 colonoscopies.

We found lower BMI to be another statistically significant predictor of perforation. Literature on the relation between BMI and risk of colonic perforation is sparse. Takahashi *et al*[20] postulated lower BMI as a predictor of pain and difficult colonic intubation during colonoscopy. Patients with low BMI may have sharper angulation of the sigmoid colon which theoretically can predispose these patients to a higher chance of mechanical injury during colonoscopy.

Increasing number of comorbidities resulted in increased risk of colonic perforation. Our findings are in congruence with other studies Gatto *et al*[13] and Arora *et al*[19] that demonstrated an increased risk of perforation with two or more co-morbidities.

Imai *et al*[21]studied the risk of perforation in patients with ESRD on hemodialysis (HD) undergoing colonoscopy. The study looked at 1106 HD patients and 13992 controls, and the authors found a higher risk of perforation among HD patients. Our study looked at patients with ESRD on hemodialysis, and also at patients with chronic kidney disease who were not on HD. There were no perforations among the 321 patients with ESRD in our cohort. We did not find any statistically significant relationship with increasing creatinine level and risk of perforation.

Low albumin has been shown to be a predictor for failure to complete colonoscopy[22]. Hypoalbuminemia is

a well-documented marker of morbidity and is a strong predictor of mortality in elderly patients[22,23]. We found low albumin level to be associated with a higher risk for perforation. It is possible that a low albumin may decrease the tensile strength of the colonic wall and also generally indicates poor health status that can theoretically predispose to higher risk for perforation.

We did not find any significant difference in the rate of perforation between colonoscopies performed by gastroenterologists or surgeons. This is in congruence with a prospective study of 13580 colonoscopies done by surgeons Wexner *et al*[24], which found that colonoscopy performed by surgeons are safe with low morbidity and mortality.

We did not find performance of biopsy or polypectomy as significant risk factors for perforation. Similar findings were noted by Arora *et al*[19], but are in contrast to Levin *et al*[14] and Misra *et al*[25] who found increased risk of perforation after polypectomy. We found that the performance of invasive procedures such as foreign body removal, hemostasis increase the risk of perforation, similar findings were noted by Arora *et al*[19]. We also found dilation as a significant risk factor for perforation in our cohort.

A potential limitation of this study is the validity of coding and capturing of all perforations. We used ICD-9 and CPT codes to capture perforations and co-morbidities. It is possible that we may have missed perforations due to incorrect coding. Also, if a patient went outside of our health care system, then some perforations would not have been reported and thus, not identified. Therefore, underestimation of the incidence of perforation is possible in this study.

In conclusion, the cumulative 7 d incidence of colonic perforation in this cohort was 0.06%. Advanced age and female gender were significantly more likely to have perforation. Increasing albumin and BMI resulted in decreased risk of colonic perforation. Having a colonoscopy indication of abdominal pain or Crohn's disease resulted in a higher risk of colonic perforation. Colonoscopies performed in inpatients and particularly the ICU setting had substantially greater odds of perforation. Biopsy and polypectomy did not increase the risk of perforation and only three perforations occurred with screening colonoscopy.

The increased risk of perforation during inpatient colonoscopy among the elderly and very elderly (> 80 years), and ICU patients is not inconsequential. On the basis of this data, we have restricted inexperienced operators (such as first year gastroenterology fellows) from performing these types of cases. Additionally those over 80 years referred for diagnostic colonoscopy should also be advised of their increased risk of perforation. By understanding which patient populations are at greatest risk for colonoscopic perforation, considering available alternatives, and adjusting patient selection criteria balancing for those at highest risk, may help to reduce the number of colonoscopic perforations.

COMMENTS

Background

This study is unique because we have used state of the art electronic medical records to collect information about risk factors which can predispose patient to a high risk of perforation. The authors have looked into multiple risk factors including but not limited to serum albumin, serum creatinine, body mass index (BMI), inpatient and outpatient colonoscopy and intensive care unit patients. Limited literature is available about the above mentioned risk factors and there propensity to cause perforation. The important findings deduced from this research can have important implication in day to day practice of colonoscopy.

Research frontiers

Authors found lower BMI to be another statistically significant predictor of perforation. Literature on the relation between BMI and risk of colonic perforation is sparse. Yuuichi postulated lower BMI as a predictor of pain and difficult colonic intubation during colonoscopy. Patients with low BMI may have sharper angulation of the sigmoid colon which theoretically can predispose these patients to a higher chance of mechanical injury during colonoscopy.

Innovations and breakthroughs

This was a retrospective cross-sectional study. Patients were retrospectively eligible for inclusion if they were 18 years and older and had an inpatient or outpatient colonoscopy procedure code in any facility within the Geisinger Health System during the period from January 1, 2002 to August 25, 2010. Data are presented as median and inter-quartile range, for continuous variables, and as frequency and percentage for categorical variables.

Peer review

This is an interesting paper on a clinically important topic and with good numbers. By understanding which patient populations are at greatest risk for colonoscopic perforation, considering available alternatives, and adjusting patient selection criteria balancing for those at highest risk, may help to reduce the number of colonoscopic perforations.

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BRIEF ARTICLE

Evaluation of enterochromaffin cells and melatonin secretion exponents in ulcerative colitis

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Abstract

AIM: To study an assessment of the number of enterochromaffin cells and expression of hydroxyindole-O-methyltransferase in colonic mucosa and urine excretion of 6-sulfatoxymelatonin in patients with ulcerative colitis.

METHODS: The study included 30 healthy subjects (group Ⅰ-C), 30 patients with ulcerative proctitis [group Ⅱ-ulcerative proctitis (UP)] and 30 patients with ulcerative colitis [group Ⅲ-ulcerative colitis (UC)] in acute phases of these diseases. The number of enterochromaffin cells (EC) was estimated in rectal and colonic mucosa. Bioptates were assembled from many different parts of the large intestine. Immunorective cells collected from various parts of the colon were counted according to the Eurovision DAKO (Dako A/S, Copenhagen, Denmark) System in the range of 10 fields in each bioptate at × 200 magnification. The level of mRNA expression of hydroxyindole-O-methyltransferase (HIOMT) in colonic mucosa was estimated with RT-PCR. Urine 6-sulfatoxymelatonin (6-HMS) excretion was determined immunoenzymatically using an IBL (IBL International GmbH, Hamburg, Germany) kit (RE 54031).

RESULTS: The number of EC cells in healthy subjects (C) was 132.40 ± 31.26. In patients of group Ⅱ (UP) and group Ⅲ (UC) the number of these cells was higher - 225.40 ± 37.35 ($P < 0.001$) and - 225.24 ± 40.50 ($P < 0.001$) respectively. Similar differences were related to HIOMT expression, which was 1.04 ± 0.36 in group C, 1.56 ± 0.56 ($P < 0.01$) in group UP and 2.00 ± 0.35 ($P < 0.001$) in group UC. Twenty-four hour 6-HMS urinary excretion was as follows: C - 16.32 ± 4.95 μg/24 h, UP - 26.30 ± 7.29 μg/24 h ($P < 0.01$), UC - 42.30 ± 12.56 μg/24h ($P < 0.001$). A correlation between number of EC cells and 6-HMS excretion was noted in all groups: r = 0.766 in patients with UP, r = 0.703 with UC and r = 0.8551 in the control group; the correlation between the results is statistically significant.

CONCLUSION: In the acute phases of both UP and UC, proliferation of EC cells and high expression of HIOMT and urine excretion of 6-HMS is noted. These changes may represent a beneficial response in the anti-inflammatory and defense mechanism.

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Key words: Enterochromaffin cells; 5-hydroxyindole-O-methyltransferase; 6-sulfatoxymelatonin; Ulcerative proctitis; Ulcerative colitis

Core tip: In the gastrointestinal tract melatonin is secreted mainly by enterochromaffin cells (EC). It appears

that hydroxyindole-O-methyltransferase (HIOMT) is enzyme determining melatonin synthesis. This indoleamine exert, antioxidant, and anti-inflammatory effects. Its synthesis can be disturbed by pro-inflammatory cytokines. The obtained results noted the proliferation of EC cells and the increase of HIOMT expression in ulcerative colitis ulcerative colitis (UC). Positive correlation between the amount of EC cells and urinary 6-sulfatoxymelatonin excretion points to the increase of melatonin secretion in the colon, but its beneficial anti-inflammatory effect was insufficient. The results indicate that the supplementation of melatonin may be useful in complex treatment of UC.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic disease with periods of exacerbation and remission. Its pathogenesis is complex and inflammatory and immune factors, as well as toxic forms of oxygen produced in this condition, play a major role in the destruction of colonic mucosa[1].

The phase of exacerbation is characterized by, inter alia, the increased production of pro-inflammatory cytokines, cell adhesion molecules and acute-phase proteins. In addition, various defense mechanisms are activated, including increased production of specific antibodies and antioxidants. Melatonin is also a potent agent in antioxidative defense through its antioxidant and anti-inflammatory properties[2,3].

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone produced by the cells of the neuroendocrine system located throughout the body amine precursor uptake and decarboxylation (APUD). It is produced from L-tryptophan, an exogenous amino acid, which is first converted in the body into serotonin with the involvement of tryptophan hydroxylase and 5-hydroxytryptophan decarboxylase. The serotonin is then converted to melatonin by arylalkylamine N-acetyltransferase (*AANAT*) and 5-hydroxyindole-O-methyltransferase hydroxyindole-O-methyltransferase (HIOMT)[4].

This indoleamine is secreted by pineal cells according to circadian rhythms, which are regulated mainly by light stimuli[5] and by enterochromaffin cells (EC) which are distributed throughout the whole gastrointestinal tract[6]. Melatonin from the gut is transported *via* the portal vein to the liver, where it is metabolized to 6-sulfatoxymelatonin (6-HMS)[7]. The amounts of urinary 6-HMS excretion are known to be indices of the synthesis and metabolism of this hormone[8].

Melatonin is secreted from the gastrointestinal tract under the influence of a range of stimuli, including nutritional factors, but the precise mechanisms regulating its release have not been recognized thoroughly. This hormone, released from enterochromaffin cells, fulfils important enteroprotective functions *via* the paraendocrine mechanism[9].

In inflammatory processes, melatonin inhibits nitric oxide and cyclooxygenase-2 synthesis and decreases the concentration of endogenous oxidants[10]. Furthermore, it induces other systems which in turn reduce oxygen free radicals: Superoxide dismutase and glutathione peroxidase. It also exerts a beneficial effect on microcirculation and tissue blood supply by inhibiting COX-2[11].

In experimental colitis in animals, the administration of exogenous melatonin decreased lipid, protein and nucleic acid oxidation, and also ameliorated epithelial damage and inflammatory infiltration[12,13]. In our studies, carried out on patients with ulcerative colitis, melatonin was demonstrated to reduce DNA damage and to stimulate the repair of hydrogen-peroxide-induced oxidative DNA damage in enterocytes[14]. Melatonin also has an influence on beneficial immune processes. Melatonin receptors have been identified on immunologically active cells. It also stimulates Th lymphocytes to produce IL-2 and IFN-γ, monocytes to produce IL-1, IL-6 and IL-12 and decreases TNF-α concentration and the expression of adhesion molecules (ICAM-1, P-selectin and MAdCAM-1)[15,16].

All these properties of melatonin can have a beneficial effect on the course of ulcerative colitis, as the mentioned pro-inflammatory factors play a significant role in the pathogenesis of this disease. Melatonin deficiency has been suggested to be one of the causes of upper digestive tract mucosal defects[17], whereas the results related to the colon are not consistent. The amount of melatonin secreted in an organism depends on the number of EC cells and their activity. Spiller *et al*[18] found an increased number of EC cells in rectal mucosa in patients with diarrhea predominant IBS. Similar observations were made by Osadchuk *et al*[19]. However, El-Salhy *et al*[20] demonstrated that patients with constipation-predominant IBS have a decreased number of EC cells in the colonic mucosa.

Some authors have tried to relate the changes observed in IBS patients to inflammatory bowel diseases but no reliable results have been obtained, either. Most of the researchers demonstrated that the number of EC cells increases in colonic mucosa in patients with ulcerative colitis[21-23]. In turn, others have detected a decreased number of EC cells in this group of patients[24,25]. These differences may result from a heterogeneous clinical and morphological evaluation of colonic mucosa.

The aim of the present study was to evaluate the number of EC cells, HIOMT expression in colonic mucosa and urinary 6-HMS excretion in patients with acute phase of ulcerative proctitis (UP) and ulcerative colitis (UC).

MATERIALS AND METHODS

Patients

The study included 30 healthy subjects (control group I (C)-aged 32.4 ± 9.8 years), 26 patients with an acute phase of ulcerative proctitis [group II (UP)-aged 31.9 ± 11. 6 years] and 30 patients with ulcerative colitis (group III (UC)-aged 33.0 ± 16.9 years). The severity of the disease was classified according to modified Mayo Clinic Score[26]. Bioptates were collected from many different parts (10-16) of the rectum and colon close to erosions and ulcerations. Histopathological activity was expressed according to the criteria of truelove and richards[27].

Methods

To determine the number of enterochromaffin cells in the bioptates, an immunohistochemical method was used with rabbit polyclonal antibodies (PSE) at a dilution of 1: 200 (Eurodiagnostica). Immunoreactive cells were counted with a computer according to the Eurovision DAKO system, in the range of 10 fields in each bioptate at × 200 magnification.

The level of mRNA expression of HIOMT was estimated with RT-PCR, and for this purpose, 50 mg of colonic tissue was used. Total RNA was isolated with trizol (Gibco) reagents, and then purified with DNase using gigagen *RNeasy* mini kit. The quantity and quality of RNA was estimated by using spectrophotometry. The obtained extract was used as a matrix in analyses of gene expression. cDNA synthesis was performed with Oligo (dT) 12-18 in an MJ Research PTG-1000 thermocycler. cDNA was obtained in reverse transcription and applied as the matrix for a PCR reaction incorporating selected fragments of the analyzed gene. The hypoxanthine phosphoribosyltransferase gene was the quantitative marker for the evaluation of the activity of the selected genes.

The reaction products were first separated on 6%-10% polyacrylamide gel stained with ethidium bromide and then subjected to densitometry to determine the reaction efficacy and the level of mRNA of the investigated genes. The expression of the investigated genes was compared to *HPRT* gene product to normalize the expression.

The urine concentration of 6-HMS was determined immunoenzymatically using an IBL kit (RE54031).

On the day of testing excretion of 6-HMS through the urine, the patients remained in a room in which white light was off at night, and they received condensed liquid meals (Nutridrink-400 mL there times a day) with a total energy value of 1800 kcal and drank 1500 mL non-carbonated isotonic mineral water. After completion of 24-hour urine collection, the urine was centrifuged and the samples were stored at -70 ℃. The 6-HMS concentration of the urine was determined immunoenzymatically using a Immuno-Biological Laboratories kit (No. RE54031). The measurements were performed by photometry at a wavelength of 450 nm using an Expert 96 reader (Biogent).

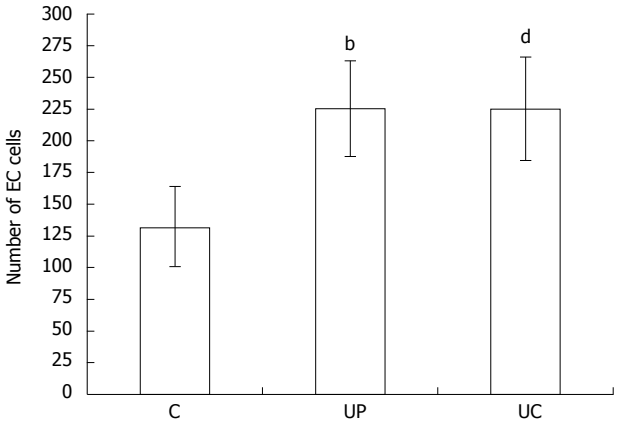

Figure 1 Number of enterochromaffin cells in colonic mucosa in healthy subjects (C), patients with ulcerative proctitis and ulcerative colitis. The number of enterochromaffin cells was counted in 10 fields in each bioptate at × 200 magnification; Healthy subjects [control group (C); n = 30]; Patients with acute phase of ulcerative proctitis (UP; n = 30); Patients with acute phase of ulcerative colitis (UC; n = 30); Differences between group C and UP $^{b}P < 0.01$; Differences between group C and UC $^{d}P < 0.01$. EC: Enterochromaffin cells.

Ethics

The study was conducted in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice. Written consent was obtained from each patient enrolled in the study and the study protocol was approved by Bioethics Committee of the Medical University of Lodz (RNN/242/06/KB).

Statistical analysis

The non-parametric Krushal-Wallis test was used to evaluate the number of enterochromaffin cells, as well as the expression of HIOMT and urinary 6-HMS excretion in the three groups: C, UP and UC. The Mann-Whitney Test was used for comparison of median values. The correlation between the number of EC cells and urinary 6-HMS extraction was estimated by the determination of Pearson's correlation coefficient and linear regression equation. The differences between the results was regarded as significant at a P value 0.05-0.001. Statistica 9.0 (StatSoft, Inc., United States) and MS Excel 2007 (Microsoft Co, United States) were used for statistical analysis.

RESULTS

The mean number of colonic EC cells in 10 fields of view in healthy subjects (group I) was 132.40 ± 31.26. However, in patients with ulcerative colitis, the number was twice as high: 225.40 ± 37.35 ($P < 0.001$) in the rectal mucosa (group II) and - 225.24 ± 40.50 ($P < 0.001$) in patients with pancolitis (group III) (Figure 1).

Similar differences were related to HIOMT expression: 1.04 ± 0.36 in healthy subjects, 1.56 ± 0.56 ($P < 0.01$) in proctitis and 2.00 ± 0.35 ($P < 0.001$) in pancolitis (Figure 2).

Urinary 6-HMS excretion in healthy subjects was 16.32 ± 4.95 and it was lower than in patients with proc-

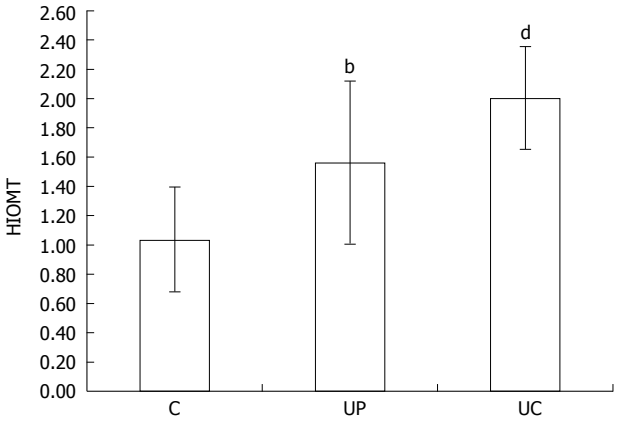

Figure 2 Expression of hydroxyindole-O-methyltransferase in colonic mucosa in healthy subjects (C), patients with ulcerative proctitis and ulcerative colitis. Healthy subjects [control group (C); *n* = 30]; Patients with acute phase of ulcerative proctitis (UP; *n* = 30); Patients with acute phase of ulcerative colitis (UC; *n* = 30); Differences between group C and UP $^{b}P < 0.01$; Differences between group C and UC $^{d}P < 0.01$. HIOMT: Hydroxyindole-O-methyltransferase.

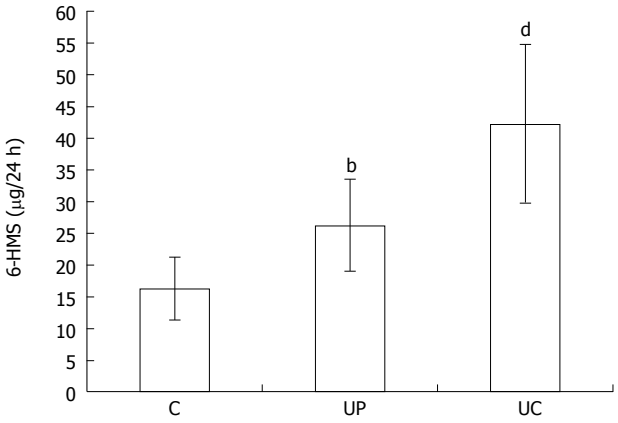

Figure 3 Urinary excretion of 6-sulfatoxymelatonin in healthy subjects (C) and patients with ulcerative proctitis and ulcerative colitis. The number of enterochromaffin cells was counted in 10 fields in each bioptate at × 200 magnification; Healthy subjects [control group (C); *n* = 30]; Patients with acute phase of ulcerative proctitis (UP; *n* = 30); Patients with acute phase of ulcerative colitis (UC; *n* = 30); Differences between group C and UP $^{b}P < 0.01$; Differences between group C and UC $^{d}P < 0.01$. 6-HMS: 6-sulfatoxymelatonin.

titis - 26.30 ± 7.29 ($P < 0.01$) and with pancolitis - 42.30 ± 12.56 ($P < 0.001$; Figure 3).

Urinary 6-HMS excretion was found to be dependent on the number of EC cells in all groups: In healthy subjects, r = 0.855, and in patients with proctitis, r = 0.766 (Figure 4A). The number of EC cells was found to have a particularly strong positive correlation with urinary 6-HMS excretion in patients with pancolitis r = 0.703 (Figure 4B).

DISCUSSION

The obtained results confirm earlier observations, which indicate that EC cell proliferation is present in the active phase of ulcerative colitis, regardless of the location of

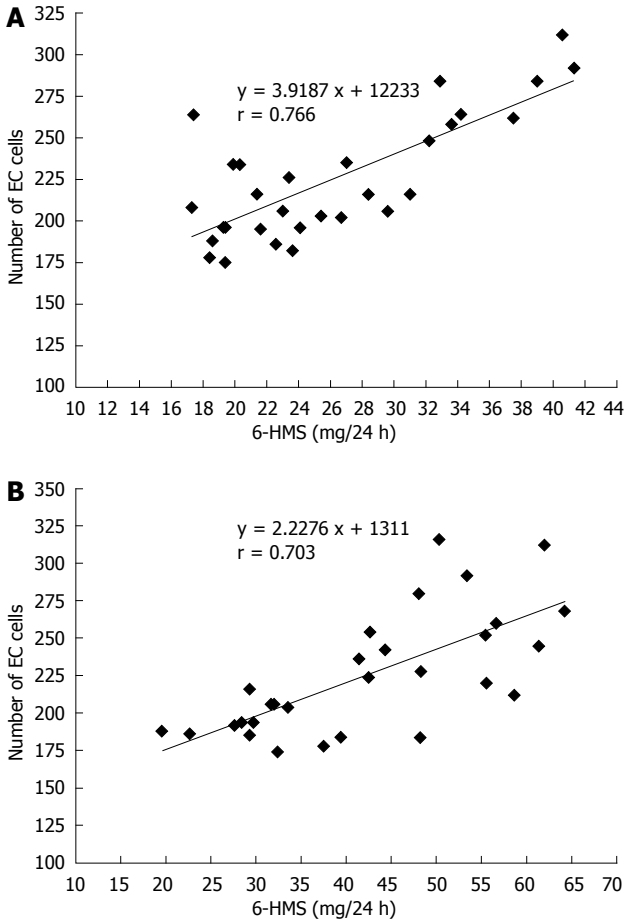

Figure 4 Correlation between the number of enterochromaffin cells and excretion of 6-sulfatoxymelatonin in patients. A: With ulcerative proctitis (group Ⅱ); B: with ulcerative colitis (group Ⅲ). The number of enterochromaffin cells was counted in 10 fields in each bioptate at × 200 magnification; y: Linear regression equation; r: Pearson's correlation coefficient; 6-HMS: 6-sulfatoxymelatonin; EC: Enterochromaffin cells.

inflammatory changes in colonic mucosa[21-23]. Additional observations found in this group of patients are firstly, an increase of HIOMT expression in the colon and secondly, a high level of urinary 6-HMS excretion. All these observations indicate increased activity of the melatonergic system in patients with ulcerative colitis. The increase of urinary 6-HMS excretion, in particular, is recognized to be the correct indicator of total melatonin production and content in the whole organism.

This pool of melatonin, which includes both the intestinal and pineal components, which representnearly the whole amount, is metabolized in the liver. Nevertheless, the positive correlation between the number of EC cells and level of urinary 6-HMS excretion indicates an increase of melatonin production mainly in the colon, as confirmed by the high HIOMT expression, compared to healthy subjects. However, it should be emphasized that melatonin catabolism both in the liver and in the colon can be modified by other factors; an excessive increase of pro-inflammatory cytokines in the colonic wall can disturb metabolism of this hormone.

Furthermore, due to the "consumption" of melato-

nin in the reaction with oxygen free radicals at the infection site, and its metabolism in the cells of the immune system, other metabolites such as 2-hydroxymelatonin, 3-hydroxymelatonin can be formed[28,29]. In turn, amino salicylates and immunosuppressive drugs applied in the treatment of ulcerative colitis exert a hepatotoxic effect and can also impair the melatonin metabolism in hepatocytes.

Due to these reasons, and probably also to the profound destruction of the colonic mucosa, decreased urinary 6-HMS excretion was observed in patients with severe ulcerative colitis. In less severe forms of the disease, these values were higher[30]. Therefore, it should be acknowledged that inflammatory processes in the colon are accompanied by increased secretion of enteral melatonin. This hormone plays a crucial enteroprotective role and its increase in ulcerative colitis is an important defense mechanism.

However, these beneficial changes in melatonin homeostasis are not sufficient to inhibit the inflammatory process and to obtain spontaneous remission of the disease. Remission requires the administration of many medications, including anti-inflammatory ones and immunosuppressants, because the pathogenesis of ulcerative colitis is complex and conditioned by numerous pro-inflammatory factors.

Serotonin is one such factor. It is secreted by the same EC cells and it is the precursor of melatonin. EC cell proliferation can, at the same time, lead to increase of serotonin secretion. The balance between serotonin and melatonin depends on the expression of enzymes regulating their synthesis and catabolism. Previous studies have not given unequivocal results. Carpuso *et al*[31] and Magro *et al*[32] found decreased serotonin concentration in colonic mucosa in patients with ulcerative colitis. However, in experimental animals with ulcerative colitis, a significant increase was observed both of EC cells and serotonin concentration in the colonic mucosa[33,34].

Regardless of the factors which contribute to the maintenance of the inflammatory process, the increase of melatonin secretion is a beneficial reaction. In our earlier studies, exogenous melatonin was also found to exert a positive effect on maintaining the remission of ulcerative colitis[35], which is the main goal of pharmacotherapy in this disease.

In conclusion, EC proliferation, HIOMT expression and increased urine excretion of 6-HMS is seen in the acute phases of ulcerative proctitis and ulcerative colitis. Consequently, the increase of enteral melatonin secretion is a beneficial response in antiinflammatory and defense mechanisms.

COMMENTS

Background

Melatonin is synthesized mainly by pinealocytes and by enterochromaffin cells in the gastrointestinal tract. This indoleamine displays endocrine and paracrine properties which account for enteroprotective action. Particular, melatonin and its metabolites are powerful antioxidants.

Research frontiers

It is known that melatonin deficiency plays an important role in the pathogenesis of certain gastrointestinal diseases, such as gastroesophageal diseases, duodenal ulcer and functional disorders, dyspepsia, irritable bowel syndrome and others. Melatonin homeostasis in inflammatory bowel diseases is not determined and the results of some researches are not unequivocal.

Innovations and breakthroughs

It is believed to be the first study of report an association between the number of enterochromaffin cells, expression of hydroxyindole-O-methyltransferase (HIOMT) and urine excretion of 6-sulfatoxymelatonin (6-HMS) in patients with ulcerative colitis.

Applications

Evaluation of 24-h urinary excretion of 6-sulfatoxymelatonin may be a useful method for the estimation of melatonin secretion and intensity of inflammatory process in colon mucosa in patients with acute phase of ulcerative colitis. During non active phase of this disease secretion of melatonin is probably decreased and the supplementation of melatonin may by useful in complex treatment to maintain the remission.

Terminology

Melatonin is secreted by enterochromaffin cells (EC) in the gastrointestinal tract under the effect of HIOMT but the mechanism regulating its release have not been recognized thoroughly. In the gastrointestinal tract melatonin fulfils important enteroprotective role *via* paraendocrine activity. Melatonin from the gut is transported *via* portal vain to the liver where is metabolized mainly to 6-HMS. The amounts of the urinary 6-HMS excretion are recognized indices of the synthesis and metabolism of this hormone.

Peer review

The authors evaluated the melatonin synthetic pathway in EC in the colonic mucosa in patients with ulcerative proctitis and ulcerative colitis. This is novel paper reporting an important role of melatonin in the injured gastrointestinal tract. The role showed that during acute phase of this disease there was a proliferation of EC cells accompanied with elevated expression of HIOMT and urinary excretion of 6-HMS. The authors concluded that the response of melatonin synthesis may account for a beneficial response against the inflammatory process.

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BRIEF ARTICLE

Diagnostic value of endothelial markers and HHV-8 staining in gastrointestinal Kaposi sarcoma and its difference in endoscopic tumor staging

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Abstract

AIM: To clarify the diagnostic values of hematoxylin and eosin (HE), D2-40, CD31, CD34, and HHV-8 immunohistochemical (IHC) staining in gastrointestinal Kaposi's sarcoma (GI-KS) in relation to endoscopic tumor staging.

METHODS: Biopsy samples (n = 133) from 41 human immunodeficiency virus-infected patients were reviewed. GI-KS was defined as histologically negative for other GI diseases and as a positive clinical response to KS therapy. The receiver operating characteristic area under the curve (ROC-AUC) was compared in relation to lesion size, GI location, and macroscopic appearances on endoscopy.

RESULTS: GI-KS was confirmed in 84 lesions (81.6%). Other endoscopic findings were polyps (n = 9), inflammation (n = 4), malignant lymphoma (n = 4), and condyloma (n = 2), which mimicked GI-KS on endoscopy. ROC-AUC of HE, D2-40, blood vessel markers, and HHV-8 showed results of 0.83, 0.89, 0.80, and 0.82, respectively. For IHC staining, the ROC-AUC of D2-40 was significantly higher (P < 0.05) than that of HE staining only. In the analysis of endoscopic appearance, the ROC-AUC of HE and IHC showed a tendency toward an increase in tumor staging (*e.g.*, small to large, patches, and polypoid to SMT appearance). D2-40 was significantly (P < 0.05) advantageous in the upper GI tract and for polypoid appearance compared with HE staining.

CONCLUSION: The diagnostic value of endothelial markers and HHV-8 staining was found to be high, and its accuracy tended to increase with endoscopic tumor staging. D2-40 will be useful for complementing HE staining in the diagnosis of GI-KS, especially in the upper GI tract and for polypoid appearance.

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Key words: Gastrointestinal Kaposi's sarcoma; Hematoxylin and eosin; CD31; CD34; D2-40; Human herpesvirus-8

Core tip: Diagnosis of gastrointestinal Kaposi sarcoma (GI-KS) is important because treatment specifics depend on the extent of the disease. Endoscopic biopsy is a definitive diagnostic method for GI-KS, but its diagnostic accuracy has not been fully studied. In the current study, receiver operating characteristic area under the curve of hematoxylin and eosin (HE) staining, lymphatic and blood vessel endothelial cell markers, and HHV-8 was found to be high (> 0.80), and its accuracy tended to increase with endoscopic tumor staging. D2-40 will be useful for complementing HE staining in the diagnosis of GI-KS, especially in the upper GI tract and for polypoid appearance.

Nagata N, Igari T, Shimbo T, Sekine K, Akiyama J, Hamada Y, Yazaki H, Ohmagari N, Teruya K, Oka S, Uemura N. Diagnostic value of endothelial markers and HHV-8 staining in gastrointestinal Kaposi sarcoma and its difference in endoscopic tumor staging. *World J Gastroenterol* 2013; 19(23): 3608-3614 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3608.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3608

INTRODUCTION

Kaposi sarcoma (KS) is a rare cancer that was highly prevalent in the early stages of the acquired immune deficiency syndrome (AIDS) endemic[1]. Although the rate of KS has shown a marked reduction since the introduction of highly active antiretroviral therapy (HAART)[1-3], KS remains the most common malignancy in patients with AIDS[4].

KS primarily involves the skin but can also involve the viscera[5-7]. Because the need for treatment and choice of treatment depend on visceral involvement[1-3,8-11], diagnosis of the gastrointestinal (GI) tract, a common site of visceral involvement[11-13], is important. Definitive diagnosis of GI-KS requires endoscopic biopsy[6,7,14-16], but GI-KS often presents on endoscopy with submucosal or small protruded appearance[17,18], which can lead to false-negative biopsy results[14-16].

Recently, immunohistochemical (IHC) staining with D2-40, CD31, CD34, and HHV-8 has been reported as useful for distinguishing cutaneous KS from other diseases[19-28]. However, no IHC studies have reported on the utility of KS diagnosis in the GI tract. In addition, it is not known how well such staining methods provide additive effects compared with HE staining alone.

With regard to the diagnosis of cutaneous KS, there may be subtle differences in the staining patterns for endothelial markers between different histologic stages (patch, plaque, and nodular) of KS[19,21,22,25]. In the GI tract, KS has various macroscopic presentations[13-15,17,18,29] and KS may affect any part of the GI tract[7,13,14,17,18]. However, the effect of IHC-positive staining in the diagnosis of GI-KS on the basis of lesion appearance has not been fully investigated.

The purpose of this study was to clarify the diagnostic value of IHC staining in the diagnosis of GI-KS and to assess the difference in accuracy between HE and IHC staining in relation to endoscopic tumor staging.

MATERIALS AND METHODS

Subjects

We retrospectively reviewed histologic slides from 103 consecutive lesions for which IHC staining was performed between 2006 and 2012 at the National Center for Global Health and Medicine (NCGM). Lesions were obtained from 41 human immunodeficiency virus (HIV)-infected patients who had not received anti-KS therapy. The institutional review board at NCGM approved this study.

Clinical factors

Sexual behavior was classified subjects into two groups: men who have sex with men (MSM); and heterosexual. $CD4^+$ cell counts and HIV-RNA viral load (VL) determined by real-time quantitative polymerase chain reaction (PCR) were reviewed within 1 mo of endoscopy. A positive result for real-time HIV-RNA was defined as $\geqslant$ 40 copies/mL. History of HAART was collected from medical records prior to endoscopy. GI symptoms were assessed by the physician who interviewed each patient. Those without GI symptoms and negative screening endoscopy were considered to be symptom-free.

Diagnosis of GI-KS

Confirmed GI-KS lesions were defined as those that fulfilled with following criteria. (1) Histologically negative biopsy for other GI diseases; (2) A positive response to KS therapy (HAART or systemic therapy of liposomal anthracycline); and (3) partial or complete resolution was confirmed on follow-up endoscopy after KS therapy (Figure 1). We usually perform endoscopy after 1 mo, 2 mo, or 6 mo of KS therapy to evaluate GI-KS regression.

All 103 lesions were suspected to be GI-KS on endoscopy, as they exhibited properties such as reddish with patches, polypoid appearance, submucosal tumor (SMT)-like lesions, and ulcerative SMT, as previously reported[13-15,17,18,29]. Therefore, IHC staining in addition to HE staining was performed.

Endoscopic assessment

Endoscopic images were taken using a high-resolution scope (model GFH260, CFH260AI; Olympus Optical, Tokyo, Japan) in all patients. We performed a biopsy using biopsy forceps (FB-240U, FB230-K, Olympus Co., Tokyo, Japan).

Size (< 10 mm or $\geqslant$ 10 mm), GI location, and macroscopic findings were assessed endoscopically. Locations of GI involvement were classified as upper GI (esophagus, stomach, and duodenum) and lower GI (ileum, colon, and rectum).

Macroscopic findings were evaluated as the presence of reddish mucosa with patches (Figure 2A), polypoid lesions (Figure 2B), submucosal tumor (SMT) (Figure 2C), and ulcerative SMT (Figure 2D), as previously reported[13-15,17,18,29]. Ulceration was defined endoscopically as a distinct, visible crater > 5 mm in diameter with a slough base.

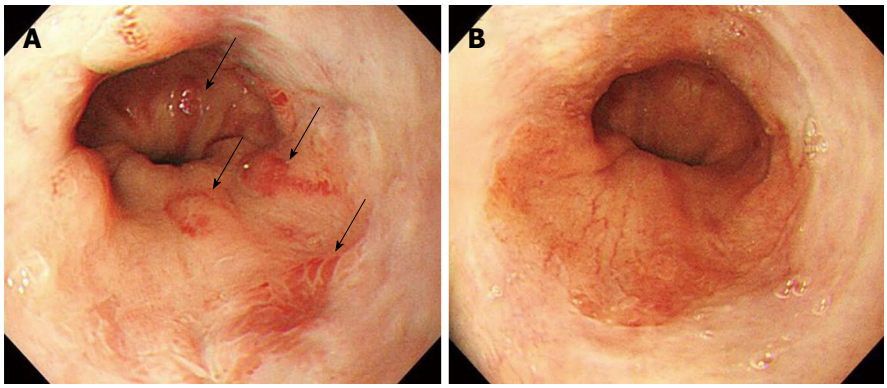

Figure 1 Confirmation of clinical response on follow-up endoscopy before and after Kaposi's sarcoma therapy. A: Gastrointestinal Kaposi sarcoma (arrows) in the esophagogastric junction before Kaposi's sarcoma (KS) therapy; B: After four months of KS therapy with liposomal anthracycline.

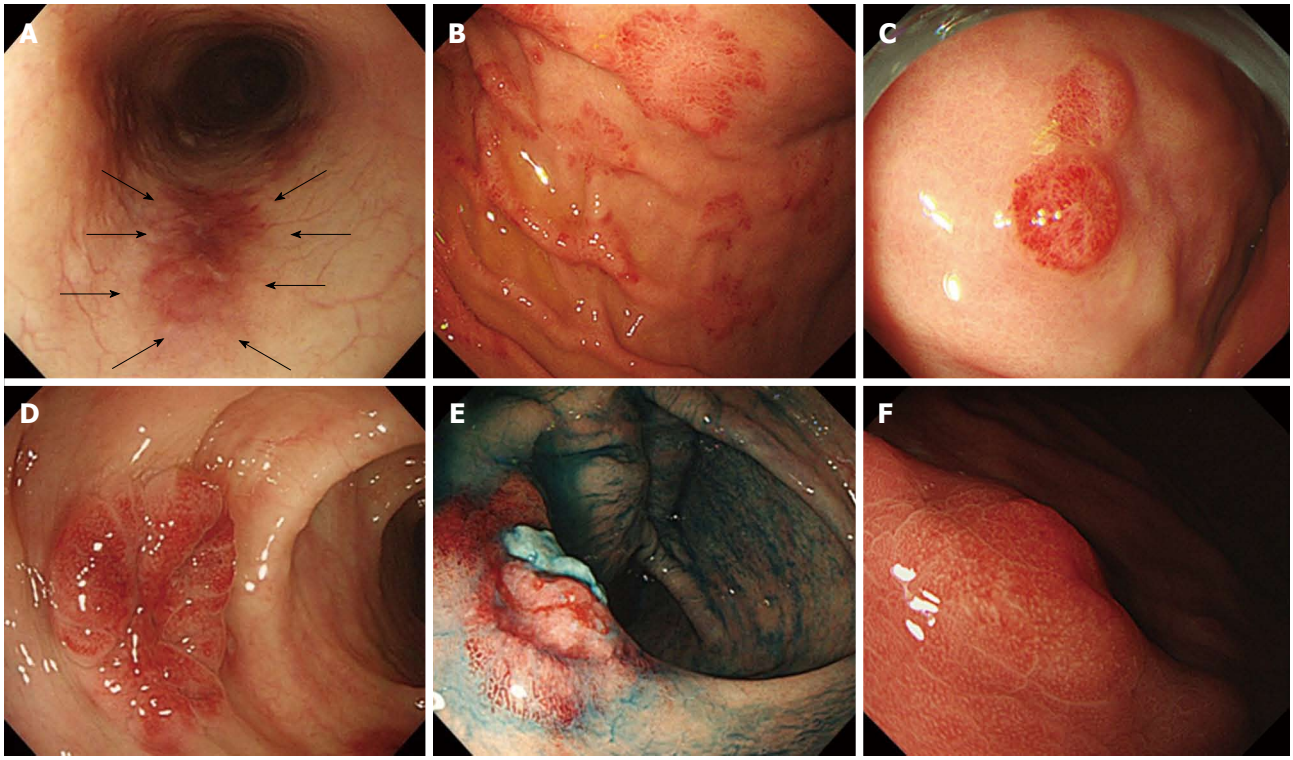

Figure 2 Gastrointestinal Kaposi sarcoma and mimicking lesions on endoscopy. A: Kaposi sarcoma, dark reddish patch (arrows) in the esophagus; B: Kaposi sarcoma, multiple patch appearance in the stomach; C: Kaposi sarcoma, small (< 10 mm) and polypoid appearance in the stomach; D: Kaposi sarcoma, submucosal tumor (SMT) appearance with large size (≥ 10 mm) in the sigmoid colon; E: Kaposi sarcoma, submucosal tumor (SMT) appearance with ulceration in the ileo-cecal valve with indigo-carmine dye; F: Hyperplastic polyps mimicking Kaposi sarcoma with small size (< 10 mm) in the stomach.

Histological assessment

The presence of proliferating spindle cells with vascular channels filled with blood cells (Figure 3A) from biopsy specimens was evaluated with HE staining by an investigator blinded to IHC staining results. IHC staining for the lymphatic vessel endothelial cell marker D2-40 (Dako North America, Carpentaria, CA) (Figure 3B) and the blood vessel endothelial cell markers CD31 (Dako North America) or CD34 (Dako North America) (Figure 3C), and the use of the mouse monoclonal antibody against HHV-8 LNA-1 (Novocastra Laboratories Ltd, Newcastle upon Tyne, United Kingdom) (Figure 3D), were also evaluated on formalin-fixed, paraffin-embedded tissue sections as previously reported[19-28]. IHC slides were evaluated at × 200 and × 400 magnification by expert GI pathologists.

Statistical analysis

To elucidate the accuracy of HE and IHC staining for the diagnosis of GI-KS, the sensitivity, specificity, positive and negative likelihood ratio (LR+ and LR-, respectively), and area under the receiver operating characteristic curve (ROC-AUC) were calculated and estimated with a 95%CI.

The difference of the ROC-AUC of the four specific stains (HE, D2-10, vessel markers, and HHV-8) was compared. Subgroup analysis was performed to identify differences in four specific stains according to gross ap-

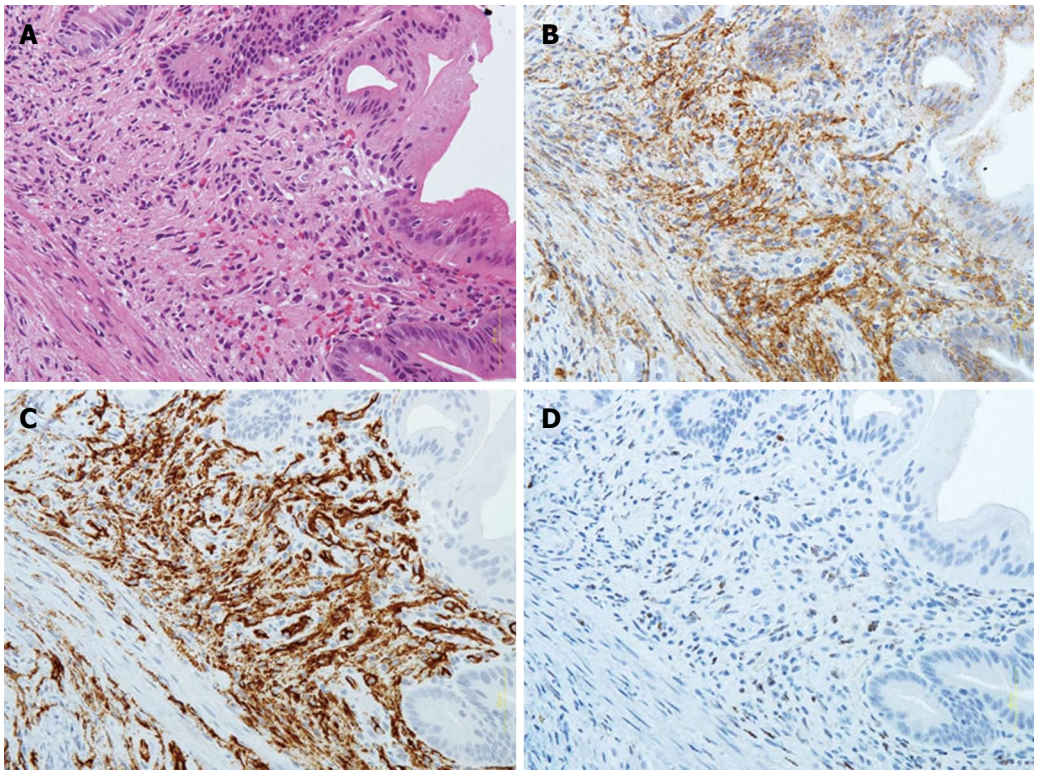

Figure 3 Pathological features of gastrointestinal Kaposi sarcoma. A: Spindle cell proliferation in the submucosa on hematoxylin and eosin (HE) staining; B: Vascular gaps are lined with endothelial cells when staining with D2-40; C: Vascular gaps are lined with endothelial cells when staining with CD34; D: Some endothelial cells are positive for human herpesvirus 8.

Table 1 Definitive diagnosis of gastrointestinal lesion from endoscopic biopsy samples (*n* = 103) *n* (%)

Diagnosis	No.
GI-KS	84
Upper GI tract	57 (67.9)
Esophagus	7 (8.3)
Stomach	38 (45.2)
Duodenum	12 (14.3)
Lower GI tract	27 (32.1)
Cecum	8 (9.5)
Ascending colon	1 (1.2)
Transverse colon	2 (2.4)
Descending colon	1 (1.2)
Sigmoid colon	7 (8.3)
Rectum	8 (9.5)
Non-KS lesion	19
Hyperplastic polyp	8
Fundic gland polyps	1
Helicobacter-associated gastritis	1
Malignant lymphoma	4
Anorectal condyloma	2
Non-specific colitis	3

GI-KS: Gastrointestinal Kaposi sarcoma.

pearances, and the ROC-AUC was compared in each group. ROC-AUC differences between HE staining and specific IHC staining in each groups were also compared.

Values of $P < 0.05$ were considered significant. All statistical analysis was performed using Stata version 10 software (StataCorp, College Station, TX).

RESULTS

Baseline clinical characteristics

All 41 HIV-infected patients were male and the HIV infection route was MSM in all cases. The median CD4 cell count (interquartile range; IQR) was 77 (33, 157) cells/mL and the median HIV viral load (IQR) was 48500 (< 40, 150000) copies/mL. There were 18 (43.9%) patients with a history of HAART. GI symptoms were noted in 10 patients (24.4%). No notable gastrointestinal bleeding or perforation, either spontaneously or after endoscopic biopsy, was noted.

Table 1 provides details on the definitive diagnosis of GI lesions. Of the 103 lesions, 84 (81.6%) were confirmed as GI-KS while the remainder were other GI lesions (19) consisted of hyperplastic polyps (8), fundic grand polyps (1), *Helicobacter*-associated gastritis (1), malignant lymphoma (4), anorectal condyloma (2), and non-specific colitis (3).

Diagnostic value of specific staining for the diagnosis of GI-KS

Sensitivity, specificity, LR+, LR-, and ROC-AUC of specific staining for the diagnosis of GI-KS are shown in Table 2. The ROC-AUC values of four specific stains (HE, D2-40, blood vessel marker, and HHV-8) were significantly different ($P < 0.01$) in the diagnosis of GI-KS (Table 2). The ROC-AUC of D2-40 staining was only significantly higher ($P < 0.05$) than that of HE staining (Table 2).

Table 2 Diagnostic value of endoscopic biopsy in gastrointestinal Kaposi sarcoma (n = 103)

KS/non-KS (84/19)	Sensitivity, % (95%CI)	Specificity, % (95%CI)	Positive LR (95%CI)	Negative LR (95%CI)	ROC area[1] (95%CI)
HE (59/1)	70.2 (59.3-79.7)	94.7 (74.0-99.9)	9.33 (1.99-43.8)[2]	0.32 (0.23-0.46)[2]	0.83 (0.75-0.90)
D2-40 (65/0)	77.4 (67.0-85.8)	100 (82.4-100)	30.8 (1.99-477)[2]	0.24 (0.16-0.35)[2]	0.89 (0.84-0.93)[a]
Blood vessel marker (68/4)	81 (70.9- 88.7)	78.9 (54.4- 93.9)	3.85 (1.6-9.24)	0.24 (0.15-0.40)	0.80 (0.70-0.90)
HHV-8 (53/0)	63.1 (51.9-73.4)	100 (82.4-100)	25.2 (1.62-391)[2]	0.38 (0.29-0.51)[2]	0.82 (0.76-0.87)

LR: Likelihood ratio; HE: Hematoxylin and eosin; HHV: Human herpesvirus. [1]Receiver operating characteristic (ROC) area is significantly (P < 0.01) different in this category; [2]LR estimated using the substitution formula. A value of 0.5 was added to all cell frequencies before calculation; [a]P < 0.05 for comparisons of lesions by HE staining.

Table 3 Diagnostic values of gastrointestinal Kaposi sarcoma in relation to size, location, and macroscopic appearances on endoscopy (n = 103)

Subgroup	Specific stain	No. of lesions (KS/non-KS)	ROC-AUC (95%CI)	P value[1]
Size	Size < 10 mm	26/7		
	HE	14/0	0.77 (0.67-0.87)	
	D2-40	16/0	0.81 (0.71-0.90)	
	Blood vessel marker	18/4	0.56 (0.34-0.78)	
	HHV-8	10/0	0.69 (0.60-0.79)	< 0.05
	Size > 10 mm	58/12		
	HE	45/1	0.85 (0.75-0.94)	
	D2-40	49/0	0.92 (0.88-0.97)	
	Blood vessel marker	50/0	0.93 (0.89-0.98)	
	HHV-8	43/0	0.93 (0.89-0.98)	< 0.01
Location	Upper GI tract	57/9		
	HE	36/0	0.82 (0.75-0.88)	
	D2-40	42/0	0.87 (0.81-0.93)[a]	
	Blood vessel marker	44/4	0.66 (0.48-0.84)	
	HHV-8	36/0	0.82 (0.75-0.88)	< 0.01
	Lower GI tract	27/10		
	HE	23/1	0.88 (0.76-1.00)	
	D2-40	23/0	0.93 (0.86-0.99)	
	Blood vessel marker	24/0	0.94 (0.88-1.00)	
	HHV-8	17/0	0.82 (0.72-0.91)	< 0.01
Macroscopic appearance	Patches	23/4		
	HE	12/1	0.64 (0.37-0.90)	
	D2-40	14/0	0.80 (0.70-0.91)	
	Blood vessel marker	15/0	0.83 (0.73-0.93)	
	HHV-8	8/0	0.67 (0.57-0.77)	< 0.01
	Polypoid	9/3		
	HE	5/0	0.78 (0.61-0.95)	
	D2-40	8/0	0.94 (0.84-1.00)a	
	Blood vessel marker	8/3	0.44 (0.34-0.55)a	
	HHV-8	4/0	0.72 (0.55-0.89)	< 0.01
	SMT	37/7		
	HE	32/0	0.93 (0.88-0.99)	
	D2-40	33/0	0.95 (0.90-0.10)	
	Blood vessel marker	33/1	0.88 (0.73-1.00)	
	HHV-8	30/0	0.91 (0.84-0.97)	0.15
	SMT with ulcer	15/5		
	HE	10/0	0.83 (0.71-0.96)	
	D2-40	10/0	0.83 (0.71-0.96)	
	Blood vessel marker	12/0	0.90 (0.80-1.00)	
	HHV-8	11/0	0.87 (0.75-0.98)	0.34

[1]P values of receiver operating characteristic (ROC) area in each category were compared. [a]P < 0.05 for the comparison with lesions by hematoxylin and eosin (HE) staining. GI-KS: Gastrointestinal Kaposi sarcoma; ROC-AUC: ROC area under the curve; SMT: Submucosal tumor.

Diagnostic value of GI-KS according to size, location, and macroscopic appearance

The ROC-AUC of four specific stains showed a tendency toward an increase in tumor staging on endoscopy (*e.g.*, small to large, flat, protruded, and SMT appearance) (Table 3). The ROC-AUC of blood vessel marker in polypoid appearance was extremely low compared with other lesions (Table 3).

The ROC-AUC of four specific stains was significantly different in size, GI tract location, appearance of patches, and polypoid lesion for the diagnosis of GI-KS (Table 3). No significant differences were noted in the ROC-AUC of four specific stains for SMT lesions (P = 0.15) or ulcerative SMT lesions (P = 0.34) (Table 3).

Comparison of the ROC-AUC between HE staining and specific staining

The ROC-AUC of the D2-40 stain was higher than that of the HE stain for lesions < 10 mm, lesions ⩾ 10 mm, upper GI tract, lower GI tract, patches, polypoids, and SMT (Table 3). Of these, upper GI tract and polypoid appearance were statistically significant (P < 0.05). The ROC-AUC of blood vessel marker or HHV-8 stain was higher than that of HE staining for lesions ⩾ 10 mm, patches, and ulcerative SMT (Table 3), with no statistical significance (P > 0.05).

DISCUSSION

Previous IHC studies have shown the utility of differential diagnosis between cutaneous KS and vascular tumors such as hemangioma, lymphangioma, hemangioendothelioma, and angiosarcoma[19-28]. However, development of vascular tumor in the GI tract is extremely rare[30]. Therefore, differential diagnosis for GI-KS can be different for cutaneous and GI tract sites. In the present study, lesions that were difficult to distinguish from GI-KS are inflammation-associated protruded lesions with reddish color. The reason for this is that GI-KS can appear as a strong reddish mucosa and vary from flat maculopapular or polypoid masses to SMT, ulceration, or bulky tumor masses on endoscopy[14,17,18,29,31].

Previous studies investigated only GI-KS cases, and

only sensitivity can be elucidated[14-16]. In the current study, the ROC-AUC values of the four IHC stains and HE stain were > 0.8, demonstrating that all had good diagnostic accuracy. However, it is not feasible in clinical practice to diagnose KS using all stains. Based on the results of this study, we conclude that D2-40 is the only stain capable of complementing HE staining.

We found that the ROC-AUC of four specific stains tended to increase with endoscopic tumor staging (*e.g.*, small to large, flat, protruded, and SMT). Previous studies, particularly those on cutaneous KS, have also shown that diagnostic accuracy varies according to tumor staging[19,21,22,25]. Although it is not feasible to apply staging classification of cutaneous KS to the evaluation of the macroscopic appearance of GI-KS, it is important--based on their results and our findings--to take tumor appearance and staging into consideration for the pathological diagnosis of KS.

We further performed subgroup analysis of four stains to reveal differences in diagnostic accuracy. No significant differences were noted in the ROC-AUC of four specific stains in SMT lesions ($P = 0.15$) and ulcerative SMT lesions ($P = 0.34$), indicating that HE staining alone is sufficient for diagnosing lesions with SMT appearance. Although we attempted to find lesions that can be better diagnosed with the addition of other IHC stains, polypoid lesions, and location of upper GI tract attained significant ROC-AUC ($P < 0.05$) scores with D2-40. The ROC-AUC of D2-40 was always > 0.8, regardless of the size, location, or macroscopic appearance of lesions, indicating its utility as an additional staining modality.

One of the characteristic findings of this study is that the ROC-AUC of the blood vessel marker for polypoid appearance was extremely low compared with other lesions. This was due to the presence of hyperplastic polyps, meaning that CD34 staining produces positive results due to vessel proliferation. This can result in higher false-positive cases ($n = 38$) and lower diagnostic accuracy of KS.

There are several limitations of the present study. First, we assessed IHC staining as positive or negative instead of using a scoring system; a semi-quantitative system might provide more accurate or available results in clinical practice. Second, positive vessel marker staining was defined as CD31- or CD34-positive because CD31 or CD34 was used by each pathologist. However, because 80% (82/103) of the lesions were examined using CD34, and because CD34 is reportedly a more accurate marker than CD31[25], we believe the results of the vessel marker staining in the present study are reliable.

In conclusion, endoscopic biopsy for diagnosing GI-KS can be performed safely. The diagnostic accuracy of HE staining, lymphatic and blood vessel endothelial cell markers, and HHV-8 was found to be high. Among these, D2-40 had the highest accuracy. The diagnostic accuracy of four specific stains tended to increase with endoscopic tumor staging. In particular, polypoid lesions and those in the upper GI tract respond well to HE staining complemented by D2-40 staining.

ACKNOWLEDGMENTS

We wish to thank Hisae Kawashiro, Clinical Research Co-ordinator, for assistance with data collection.

COMMENTS

Background

Diagnosis of Kaposi sarcoma (KS) involving the gastrointestinal (GI) tract is important because treatment specifics depend on the extent of the disease. Definitive diagnosis of GI-KS requires endoscopic biopsy with hematoxylin and eosin (HE) or immunohistochemical (IHC) staining. IHC staining for the differential diagnosis of cutaneous disease has been extensively studied, but the diagnostic value of GI-KS remains unknown.

Research frontiers

GI-KS often presents various endoscopic appearances, which can lead to false-negative biopsy results. Furthermore, the difference in accuracy of IHC staining in relation to endoscopic appearances has not been fully investigated. In the current study, the authors demonstrate the diagnostic value of IHC staining for GI-KS and to assess the difference in accuracy between HE and IHC staining in relation to endoscopic tumor staging.

Innovations and breakthroughs

Previous reports have highlighted the accuracy of IHC for diagnosing cutaneous KS. This is the first study to report that the receiver operating characteristic area under the curve (ROC-AUC) of HE staining, lymphatic and blood vessel endothelial cell markers, and HHV-8 for diagnosing GI-KS was found to be high (> 0.80), and its accuracy tended to increase with endoscopic tumor staging. In particular, polypoid lesions and those in the upper GI tract respond well to HE staining complemented by D2-40 staining.

Applications

In the current study, the ROC-AUC values of the four IHC stains and HE stain were good, but it is not feasible in clinical practice to diagnose KS using all stains. Based on the results of this study, the authors conclude that D2-40 is the only stain capable of complementing HE staining.

Terminology

The ROC is a diagnostic testing modality that presents its results as a plot of sensitivity *vs* 1-specificity (false-positive rate). The ROC-AUC indicates the probability of a measure or predicted risk being higher for patients with disease than for those without disease.

Peer review

This is an excellent paper describing novel findings of IHC in diagnosing GI-KS.

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BRIEF ARTICLE

Gastric precancerous lesions are associated with gene variants in *Helicobacter pylori*-susceptible ethnic Malays

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Abstract

AIM: To identify genes associated with gastric precancerous lesions in *Helicobacter pylori* (*H. pylori*)-susceptible ethnic Malays.

METHODS: Twenty-three Malay subjects with *H. pylori* infection and gastric precancerous lesions identified during endoscopy were included as "cases". Thirty-seven Malay subjects who were *H. pylori* negative and had no precancerous lesions were included as "controls". Venous blood was collected for genotyping with Affymetrix 50K Xba1 kit. Genotypes with call rates < 90% for autosomal single nucleotide polymorphisms (SNPs) were excluded. For each precancerous lesion, associated SNPs were identified from Manhattan plots, and only SNPs with a χ^2 *P* value < 0.05 and Hardy Weinberg Equilibrium *P* value > 0.5 was considered as significant markers.

RESULTS: Of the 23 *H. pylori*-positive subjects recruited, one sample was excluded from further analysis due to a low genotyping call rate. Of the 22 *H. pylori*-positive samples, atrophic gastritis only was present in 50.0%, complete intestinal metaplasia was present in 18.25%, both incomplete intestinal metaplasia and dysplasia was present in 22.7%, and dysplasia only was present in 9.1%. SNPs rs9315542 (*UFM1* gene), rs6878265 (*THBS4* gene), rs1042194 (*CYP2C19* gene) and rs10505799 (*MGST1* gene) were significantly associated with atrophic gastritis, complete intestinal metaplasia, incomplete metaplasia with foci of dysplasia and dysplasia, respectively. Allele frequencies in "cases" *vs* "controls" for rs9315542, rs6878265, rs1042194 and rs10505799 were 0.4 *vs* 0.06, 0.6 *vs* 0.01, 0.6 *vs* 0.01 and 0.5 *vs* 0.02, respectively.

CONCLUSION: Genetic variants possibly related to gastric precancerous lesions in ethnic Malays susceptible to *H. pylori* infection were identified for testing in subsequent trials.

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Key words: Gastric precancerous lesions; Gene polymorphisms; Genome-wide association; *Helicobacter pylori*; Malays

Core tip: Gastric cancer and its precancerous lesions are exceptionally rare among ethnic Malays. Gene variants may be associated with precancerous lesions in *Helicobacter pylori*-susceptible Malays. Genome-wide association was performed to identify gene variants in Malays with a spectrum of gastric precancerous lesions. Results indicated that at different phases of the Correa cascade, different gene variants were manifest, but they followed a pattern of progression similar to their histological and clinical stages. It is possible that, in addition to histological staging, gene variant markers may serve to identify different phases of gastric cancer progression in the near future.

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INTRODUCTION

Gastric cancers are thought to arise from a cascade of histological changes or precancerous lesions (atrophic gastritis, intestinal metaplasia and dysplasia) before developing into full-blown malignancy[1]. In Japan, studies have shown that surveillance of these precancerous lesions is associated with increased detection of early gastric cancers and improved survival rates[2,3].

These precancerous lesions are associated with *Helicobacter pylori* (*H. pylori*) infection acquired since childhood[4]. In populations with a high prevalence of *H. pylori* infection, including those in China and Japan, precancerous lesions can be detected in up to 80% of adults[5]. Eradication of *H. pylori* infection at this stage has not been shown to be effective in these high risk populations[6].

Ethnic Malays residing in the north-eastern region of Peninsular Malaysia (state of Kelantan) have an exceptionally low prevalence of *H. pylori* infection[7,8]. Exact reasons for this low prevalence are unknown, but it could be a combination of unique environmental, host and strain virulence factors shaped by the population's evolutionary history[9-12]. Due to the extremely low acquisition of *H. pylori* infection, gastric cancer and its precancerous lesions are extremely rare in this population[13-15].

In a survey of 234 subjects undergoing upper endoscopy in a tertiary hospital from the state of Kelantan, the reported rate of atrophic gastritis was 42.3% and intestinal metaplasia was present in 7.7% (14/234) of all biopsies, but was only present in 1.4% (2/146) of the ethnic Malays[15]. This low rate of gastric precancerous lesions observed was a result of a low prevalence of *H. pylori* infection in the studied population of only 6.8%. As shown in a multivariable analysis, the risk of intestinal metaplasia and dysplasia was only significant in the presence of *H. pylori* infection[15].

A minority of this Malay population is genetically susceptible to *H. pylori* infection, and *DCC* gene polymorphism has recently been found to be responsible[16]. An aberrant methylation of this tumor suppressor gene has been observed to occur in the course of gastric carcinogenesis[17]. As such, this population may also be genetically susceptible to the development of gastric precancerous lesions.

The current study aimed to determine the gene polymorphisms associated with gastric precancerous lesions in the Malay population from north-eastern region of Peninsular Malaysia using the genome-wide association approach.

MATERIALS AND METHODS

Study subjects

Only those ethnic Malay subjects (age range 20-80 years) whose gastrointestinal symptoms required upper endoscopy were screened for study eligibility. To avoid ascertainment bias, subjects had upper gastrointestinal symptoms (including dyspepsia and/or abdominal discomfort) and required upper endoscopy to exclude gastro-duodenal diseases before being included into the study.

All Malay subjects included in the study were born in the state of Kelantan, had resided within the region for at least 3 generations and were from different families but had similar socio-economic and socio-cultural backgrounds. Subjects positive for *H. pylori* infection according to a urease test and histology and with gastric precancerous lesions identified during endoscopy were categorized as "cases", while those negative for *H. pylori* infection and precancerous lesions were categorized as "controls". "Cases" and "controls" were matched for age and gender. Subjects satisfying the above inclusion criteria were recruited into the study. Exclusion criteria included an intake of antibiotics 3 mo prior to the upper endoscopy test, upper gastrointestinal bleeding, a positive family history of *H. pylori* infection and gastric cancer, a previous history of *H. pylori* infection and chronic psychiatric and medical conditions, including cancer. Informed consent was obtained from all subjects prior to their enrolment into the study.

Cases with *H. pylori* infection and positive for precancerous lesions were extremely limited in number due to an exceptionally low rate of *H. pylori* infection among ethnic Malays. Only 23 Malay subjects were eventually included as "cases". A larger sample size for the "controls" was sought to compensate for the low sample size in "cases". Furthermore, stringent criteria were set to ensure that only subjects of similar age, socio-economic and socio-cultural backgrounds were included in the study. From a total of 45 screened subjects, 37 Malay subjects

were recruited as "controls" with eight subjects being excluded as they did not meet the inclusion criteria, they did not give consent or blood samples were poor.

The study was approved by the Human Research and Ethics Committee of Universiti Sains Malaysia (USM).

Endoscopic diagnosis and histological definitions of precancerous lesions

All upper endoscopies (model GIF-140 and GIF-160; Olympus Medical Systems, Tokyo, Japan) during this period were performed by one endoscopist with at least 5 years' experience. If needed, patients were sedated accordingly. Subjects who did not stop proton pump inhibitors 2 wk before endoscopy, those who had received antibiotics prior to study, and patients who had upper gastrointestinal bleeding shortly before the study were excluded.

Endoscopic findings of gastritis and atrophy were recorded and classified based on established Sydney criteria[18] and Atrophy Club criteria[19]. Biopsies were taken using standard biopsy forceps at the antrum, incisura and body. A minimum of 2 to 4 biopsies (size between 2 to 4 mm) were taken in each sites and these gastric biopsies, preserved in formalin containers, were transported to the pathology laboratory on the same day.

Only one histopathologist was involved in reviewing all the slides. All biopsies were stained with routine hematoxylin and eosin (HE) stain followed by Alcian blue-periodic acid Schiff stain for the detection of intestinal metaplasia. The Warthin Starry stain would be used in sections where the *H. pylori* bacterium was not detected in the routine HE stain.

Chronic atrophic gastritis was identified based on the updated 1994 Sydney system[20] and Atrophy Club definitions[19]. Intestinal metaplasia was identified by replacing glandular epithelium with goblet cells[21]. Intestinal metaplasia was classified into complete or incomplete types. Complete type resembled the small intestinal phenotype with well-formed goblet cells while incomplete type resembled the colonic phenotype with irregular mucin droplets and absence of a brush border. Dysplasia was identified by epithelium disarray and increased nucleocytoplasmic ratio[22].

For the purpose of the genotyping study, subjects were grouped as follows: atrophic gastritis only, complete intestinal metaplasia, incomplete metaplasia with foci of dysplasia, or dysplasia only.

Genomic DNA preparation

All recruited subjects were called up by one of the investigators (SM) to have 1 mL of venous blood taken during the study day. Unlike conventional methods of DNA extraction, 1 mL of blood was sufficient for the commercially available kits. The blood was collected in an EDTA tube and was transported immediately to a facility (Human Genome Centre, USM, Kubang Kerian, Malaysia) to be stored at 4 ℃. Subsequently, DNA for all recruited cases and controls was isolated using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany).

Genotyping with Affymetrix 50K Xba1

The isolated DNA from all recruited cases (n = 23) and controls (n = 37) were processed and genotyped using Affymetrix 50k Xba1 array (Affymetrix, United States) following the instructions provided in the Affymetrix GeneChip Human Mapping 100K Assay Manual[23]. Genotypes with call rates < 90% for autosomal single nucleotide polymorphisms (SNPs) were excluded. SNPs that had a minor allele frequency < 5%, that failed to genotype in > 5% of samples, or had a Hardy-Weinberg Equilibrium (HWE) P-value < 0.5 were also excluded from the analysis.

Statistical analysis

Genotype calling to assess the normalization of the SNPs was performed with the Bayesian Robust Linear Model with Mahalanobis distance classifier (BRLMM) algorithm from the Affymetrix® Genotyping Console™ software version 4.0 (Affymetrix, United States). Quality control for genetic markers was assessed using the Genotype filtering tool in the SVS Golden Helix Bioinformatics Tools version 7.4 (Golden Helix Inc., Bozeman, MT, United States).

Association was evaluated for every single SNP in each gene with SVS Golden Helix Bioinformatics Tools. False Discovery Rate, and Bonferroni adjustments were used for multiple-testing corrections. A Manhattan plot for each phenotype was generated to determine SNPs with the highest significant value associated with that phenotype using SVS Golden Helix Bioinformatics Tools (version 7.4). A significant genomic threshold of 3×10^{-7} in Manhattan plots was set in this study and a χ^2 P value for each SNP was calculated based on Fisher's exact χ^2 test. For each type of precancerous lesion studied, of which a group of associated SNPs were identified from Manhattan plots, only a SNP with χ^2 P value < 0.05 and HWE P value > 0.5 was considered as a significant marker.

RESULTS

Of the 23 *H. pylori*-positive subjects recruited, one sample was excluded from further analysis due to a low genotyping call rate (< 90%). The mean age of the remaining 22 "cases" was 56.5 ± 16.5 years, and was 53.2 ± 15.2 years for the 37 "controls". Cases were 54.5% (12/22) male compared with 50% (17/37) in "controls". Of subjects positive for *H. pylori* infection (cases), atrophic gastritis only was present in 50.0% (11/22), complete intestinal metaplasia was present in 18.2% (4/22), both incomplete intestinal metaplasia and dysplasia was present in 22.7% (5/22) and dysplasia only was present in 9.1% (2/22). None of the gastric precancerous lesions were present in subjects negative for *H. pylori* infection (controls).

In 10 "cases" with atrophic gastritis only, compared with controls, 26 SNPs were above the significant ge-

Table 1 Single nucleotide polymorphisms associated with atrophic gastritis among Malays with *Helicobacter pylori*

rs ID	Chromosome	Gene	Minor allele	Allele frequency		χ^2	HWE
				Cases	Control	*P*-value[1]	*P*-value
rs2614074	8 p21.1	*PNOC*	B	0.409	0.062	0.008	0.983
rs10504944	8 q22.1	*GDF6*	A	0.5	0	0.034	0.516
rs9315542	13 q13.3	*UFM1*	B	0.409	0.064	0.007	0.994
rs4943552	13 q13.3	*UFM1*	A	0.318	0.075	0.040	0.815
rs489977	18 q12.3	*KC6*	A	0.181	0	0.064	0.752

rs ID: The unique ID for the identified single nucleotide polymorphisms; HWE: Hardy-Weinberg Equilibrium. [1]All markers were run using the FAMHAP (Haplotype Association Analysis) program. The *P* value represents the simulated overall significance for the particular marker corrected for multiple testing and $P < 0.05$ was considered statistically significant.

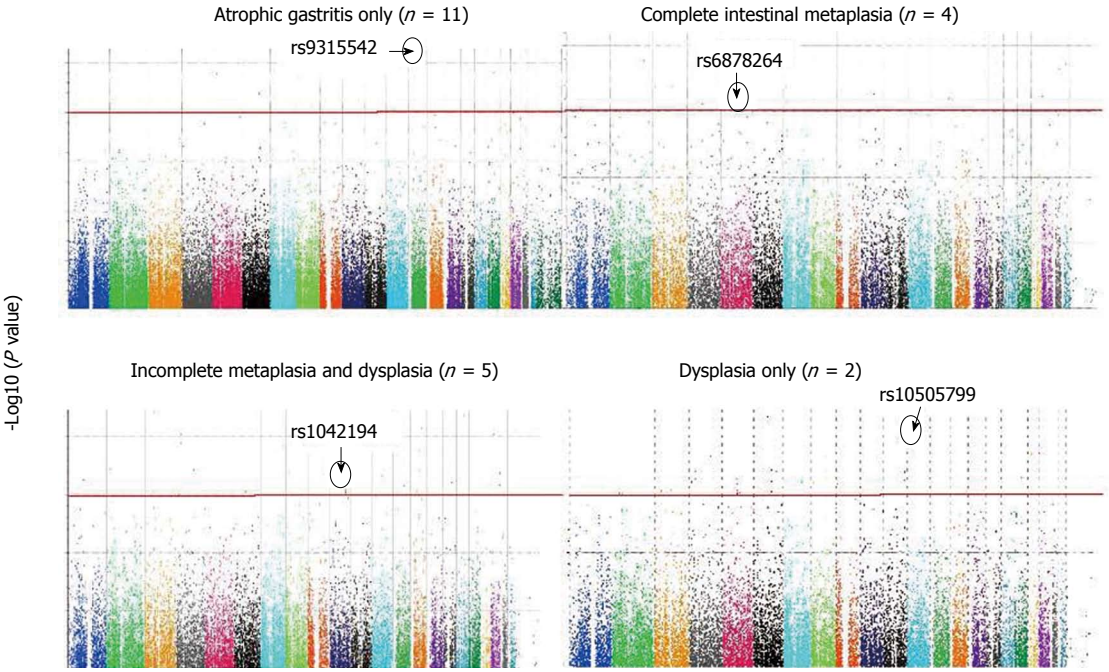

Figure 1 Manhattan plots for different gastric precancerous lesions (phenotype) in susceptible Malays with *Helicobacter pylori*. Red line indicates genomic threshold (3×10^{-7}) set to determine single nucleotide polymorphisms (SNPs) in Hardy-Weinberg Equilibrium associated with the studied phenotype. The most significant SNP, as determined by the χ^2 *P* value, for each phenotype is shown by an arrow.

nomic threshold. Five of the identified 26 SNPs were in HWE, of which rs9315542, located in chromosome 13 q13.3 (*UFM1* gene), was the most significant associated SNP (χ^2 *P* value = 0.007) (Table 1, Figure 1). The allele frequency for rs9315542 in cases *vs* controls was 0.4 *vs* 0.06.

In 4 "cases" with intestinal metaplasia only, compared with controls, 13 SNPs were above the genomic threshold and were in HWE, of which rs6878264, located in intron 4 of the *thrombospondin 4 (THBS4)* gene, was the most significant associated SNP (χ^2 *P* value = 0.01) (Table 2, Figure 1). The allele frequency for rs6878264 in cases *vs* controls was 0.6 *vs* 0.01.

In 6 "cases" with both intestinal metaplasia and dysplasia, compared with controls, 17 SNPs were above the genomic threshold and in HWE, of which rs1042194, located in exon 8 of the *CYP2C19* gene, was the most significant associated SNP (χ^2 *P* value = 0.00536) (Table 3, Figure 1). The allele frequency for rs1042194 in cases *vs* controls was 0.6 *vs* 0.01.

Finally, in 2 "cases" with dysplasia only, compared with controls, 2 SNPs were above the genomic threshold and in HWE, of which rs10505799, located in chromosome 12p12.3 (*MGST1* gene), was the most significant associated SNP (χ^2 *P* value = 0.006) (Table 4, Figure 1). The allele frequency for rs10505799 in cases *vs* controls was 0.5 *vs* 0.02.

DISCUSSION

In this Malay population with an extremely low risk of *H. pylori* infection, gastric cancer and its precancerous lesions are very rare. However, in the current study of subjects susceptible to *H. pylori* infection and those who developed precancerous lesions, certain gene polymorphisms were found to be more commonly associated with precancerous lesions. Notwithstanding the low sample size, resulting from the extremely rare occurrence of gastric

Table 2 Single nucleotide polymorphisms associated with intestinal metaplasia among Malays with *Helicobacter pylori*

rs ID	Chromosome	Gene	Minor allele	Allele frequency		χ^2	HWE
				Cases	Control	*P*-value	*P*-value
rs1166704	1p31.1	*NEXN*	B	0.375	0.031	0.0694	0.654
rs2191508	2q32.3	*SLC39A10*	A	0.081	0.375	0.297	0.591
rs1992736	3p24.3	*TBC1D5*	A	0.750	0.193	0.192	0.78
rs10511297	3q13.13	*CD96*	A	0.375	0.030	0.171	0.659
rs2615485	4q22.1	*DSPP*	A	0.750	0.136	0.190	0.625
rs2434316	5q14.1	*THBS4*	A	0.750	0.257	0.118	0.743
rs6878264	5q14.1	*THBS4*	B	0.625	0.010	0.010	1.000
rs7800141	7p15.3	*DNAH11*	B	0.666	0.096	0.154	0.717
rs4746259	10q22.2	*PPIAL4G*	B	0.750	0.015	0.062	0.794
rs9300471	13q32.2	*FARP1*	A	0.375	0.030	0.145	0.659
rs1881344	16p13.2	*C16orf68*	A	0.375	0.030	0.078	0.659
rs2253429	20p13	*SIRPB1*	B	0.375	0.030	0.0119	0.659
rs2834681	21q22.12	*RUNX1*	B	0.250	0	0.0623	0.859

rs ID: The unique ID for the identified single nucleotide polymorphisms; HWE: Hardy-Weinberg Equilibrium.

Table 3 Single nucleotide polymorphisms associated with intestinal metaplasia and dysplasia among Malays with *Helicobacter pylori*

rs ID	Chromosome	Gene	Minor allele	Allele frequency		χ^2	HWE
				Cases	Control	*P*-value	*P*-value
rs10493872	1p21.3	*ABCD3*	A	0.375	0.062	0.00601	0.518
rs10510792	3p14.3	*DNAH12*	A	0.25	0.030	0.341	0.728
rs2889259	4p14	*KIAA1239*	A	0.375	0.015	0.0623	0.724
rs6837437	4p14	*KIAA1239*	A	0.375	0.015	0.0623	0.728
rs10498879	6q13	*RIMS1*	A	0.75	0.196	0.160	0.629
rs4073894	7q22.1	*LHFPL3*	A	0.375	0.015	0.0623	0.728
rs10487929	7q35	*CNTNAP2*	B	0.25	0.031	0.260	0.724
rs10503727	8p21.2	*SLC25A37*	B	0.75	0.183	0.151	0.908
rs2251417	10q21.3	*ANXA2P1*	A	0.375	0.045	0.579	0.591
rs1042194	10q23.33	*CYP2C19*	B	0.625	0.011	0.00536	0.972
rs10506855	12q21.31	*CCDC59*	B	0.375	0.046	0.0269	0.585
rs10492652	13q22.1	*KLF12*	B	0.25	0.015	0.0623	0.797
rs1565946	14q23.1	*SLC35F4*	A	0.625	0.156	0.0151	0.658
rs10483683	14q23.1	*SLC35F4*	B	0.625	0.181	0.314	0.964
rs10483837	14q24.2	*RGS6*	B	0.5	0.031	0.171	0.585
rs245615	16q21	*CDH8*	A	0.5	0.140	0.183	0.844
rs2058879	19q13.32	*IGFL2*	A	0.375	0.015	0.0623	0.728

rs ID: The unique ID for the identified single nucleotide polymorphisms; HWE: Hardy-Weinberg Equilibrium.

precancerous lesions in this population, the current study, with the use of the genome-wide association approach, allowed identification of genetic markers that can be tested in a larger cohort in the near future[24].

In the 50% of *H. pylori*-infected subjects with atrophic gastritis, the earliest lesion in the Correa cascade, rs9315542, located in chromosome 13 q13.3 (*UFM1* gene), was the identified marker. A recently identified expressed protein, ubiquitin-fold modifier 1 or *UFM1*, is a member of a large family of ubiquitin-like proteins or Ubls[25]. Ubiquitin, a small protein, is associated with the process of "ubiquitination", a target of proteins for degradation by the proteasome. At the moment, the exact cellular functions of proteins modified by *UFM1* remain elusive. A recent report indicated that components of the *UFM1* conjugation pathway are highly expressed in the beta cells of the pancreas and some other protein secretory tissues[26]. In the same report, *UFM1* conjugate prevented endoplasmic reticulum (ER) stress-induced apoptosis. While *UFM1* in gastric tissue has not been investigated, it is known that gastric mucosa secretes a number of peptides and hormones, including pepsinogen and ghrelin, whose levels are reduced in atrophic gastritis[27]. Speculatively, *UFM1* may be a marker of the secretory status of the gastric mucosa, similar to pepsinogen, and remains to be tested and validated.

Complete-type or type I intestinal metaplasia, considered as the benign version compared with the incomplete-type, was present in 18.2% of *H. pylori*-infected subjects. In these subjects, rs6878264 located in intron 4 of the *THBS4* gene, was the identified marker. THBS4 is a member of the THBS protein family, a glycoprotein in the extracellular matrix, which mediates cell-to-cell and cell-to-matrix interactions. Although the exact physiological functions of THBS4 are unknown, the published literature indicate that it promotes neurite outgrowth,

Table 4 Single nucleotide polymorphisms associated with dysplasia among Malays with *Helicobacter pylori*

rs ID	Chromosome	Gene	Minor allele	Allele frequency		χ^2	HWE
				Cases	Control	*P*-value	*P*-value
rs10505799	12p12.3	*MGST1*	B	0.5	0.016	0.006	0.855
rs10498391	14q21.2	*FSCB*	B	0.5	0	0.069	0.928

rs ID: The unique ID for the identified single nucleotide polymorphisms; HWE: Hardy-Weinberg Equilibrium.

stimulates proliferation of erythroid cells, skin fibroblasts and kidney epithelial cells, as well as myoblast adhesion and interaction with other extracellular matrix proteins[28-30]. Recently, THBS4 has been found to be associated with gastric adenocarcinomas especially of the diffuse type[31]. While *H. pylori* infection is associated with atrophic gastritis and intestinal metaplasia, it is not commonly associated with diffuse-type gastric adenocarcinoma[32]. Complete-type intestinal metaplasia represents a reparative process of the epithelium following *H. pylori*-induced gastritis, and in this context, *THBS4* may act as an early proliferative marker but may have a more aggressive pro-oncogenic role in advanced stages. Again, this is speculative in the absence of any published studies, but it is a worthwhile marker for further studies.

Incomplete type intestinal metaplasia is more advanced compared with complete type, and therefore is more closely associated with dysplasia. In 22.7% of cases with both incomplete type intestinal metaplasia and dysplasia, rs1042194 located in exon 8 of *CYP2C19* gene was the identified marker. Cytochrome (CYP) P450 2C19, one of the isoforms of the CYP enzyme (phase I detoxification enzyme), plays an important role in metabolism of drugs and also detoxification of potential carcinogens[33]. Several studies indicated that *CYP2C19* gene polymorphism is associated with increased cancer susceptibility including hepatocellular carcinoma, and lung, esophageal and gastric cancer, especially in patients having a poor metabolizer (PM) genotype[34-36]. A study from Malaysia found that the PM genotype was uncommon among ethnic Malays (5.9%), compared with Chinese (19.1%) and Indians (10%)[37]. This may be one of the reasons for reduced susceptibility to gastric cancer and its precancerous lesions among ethnic Malays. The finding of *CYP2C19* in incomplete type intestinal metaplasia and dysplasia in a group of Malay subjects susceptible to *H. pylori* infection is therefore important and merits further study.

Dysplasia, a histological stage with high risk of malignant transformation, was present in only 9.1% or 2/23 subjects infected with *H. pylori*. Compared with controls, rs10505799 located in chromosome 12p12.3 (*MGST1* gene) was found to be the SNP marker associated with dysplasia. Microsomal glutathione S-transferase 1 (*MGST1*) is one of the glutathione S-transferase (GST) family of enzymes, and GSTs are phase II detoxification enzymes, which, similar to CYP enzymes, are involved in the detoxification of potential carcinogens[38,39]. Recently, *MGST1* gene polymorphism was found to be involved in colorectal carcinogenesis in the Chinese population but there is no data as yet on gastric cancer[40]. However, since *MGST1* and *CYP2C19* are both carcinogen detoxification enzymes, with evidence supporting their involvement in gastrointestinal tract carcinogenesis, the role of *MGST1* in gastric precancerous lesions is likely to be valid. The limited number of cases with dysplasia in the current study means that the results need to be interpreted cautiously, but the potential of *MGST1* as a marker for dysplasia should not be disregarded.

There are a number of studies on gene polymorphisms associated with gastric precancerous lesions in high prevalence populations, but our study covered the entire spectrum of the Correa cascade in a population with an extremely low burden of gastric cancer and *H. pylori* infection. Development of gastric cancer is thought to involve multi-step carcinogenesis and follows a progressive pattern of pathological stages described by Correa. Our results indicated that, at different phases of the Correa cascade, different gene variants are manifest, but they follow a pattern of progression similar to their histological and clinical stages. During the stage of atrophic gastritis, *UFM1* expression reflects the secretory status of epithelium. With early development of intestinal metaplasia, *THBS4* acts as a proliferative marker but at more advanced stages, incomplete intestinal metaplasia and dysplasia involve polymorphisms of detoxification enzymes, *CYP2C19* and *MGST1*. Based on the current study, it is possible that, in addition to histological staging, gene variant markers may also serve to identify different phases of progression of gastric cancer in the near future. Recently, epigenetic silencing of *FOXD3* has been shown to be an early event in gastric carcinogenesis[41] and, together with genomic changes, it would allow a greater understanding of the pathogenesis of gastric cancer.

We acknowledge from the outset that the current study, based upon a genome-wide approach, was extremely limited in sample size, as gastric precancerous lesions are extremely rare among ethnic Malays from the north-eastern region of Peninsular Malaysia. In this respect, bioinformatics and statistical approaches were taken into consideration for a more reliable analysis of the data. To reduce false-positive results, a more stringent significance threshold of 3×10^{-7} was set for Manhattan plots in the current study. Only SNPs in HWE *P*-value > 0.5 were selected to reduce occasionality. In addition to being long-term residents within the studied region, cases and controls were similar in age, socio-cultural and economic backgrounds. The current study only identified

SNPs associated with gastric precancerous lesions, and further validation studies are in progress to confirm their regulatory role in carcinogenesis.

In conclusion, we have shown that, compared with controls, susceptible ethnic Malays with *H. pylori* infection expressed different SNP markers at different spectrums of gastric precancerous lesions. These markers may allow efficient screening of precancerous lesions in larger cohorts of *H. pylori*-infected individuals.

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COMMENTS

Background

Gastric cancer and its precancerous lesions are exceptionally rare among ethnic Malays. Gene variants may be associated with precancerous lesions in *Helicobacter pylori* (*H. pylori*)-susceptible Malays.

Research frontiers

In a case-control study, genome-wide association was performed to identify gene variants in the Malay population with a spectrum of *H. pylori*-associated gastric precancerous lesions.

Innovations and breakthroughs

Results indicated that at different phases of the Correa cascade, different gene variants were manifest, but they followed a pattern of progression similar to the histological and clinical stages.

Applications

It is possible that, in addition to histological staging, gene variant markers may also serve to identify different phases of progression of gastric cancer in the near future.

Terminology

The genome-wide approach utilises microarray technology to identify thousands of single nucleotide polymorphisms (SNPs). Using novel bioinformatics and statistical approaches, the association between SNPs and the studied disease can be determined reliably.

Peer review

Current study indicates that different gene variants exist that reflect different stages of progression during different spectrums of gastric carcinogenesis. These gene variants, appropriately confirmed in later studies, may be useful markers, in addition to histological staging, of gastric precancerous lesions.

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BRIEF ARTICLE

XPC Lys939Gln polymorphism, smoking and risk of sporadic colorectal cancer among Malaysians

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Abstract

AIM: To investigate the risk association of xeroderma pigmentosum group C (*XPC*) Lys939Gln polymorphism alone and in combination with cigarette smoking on colorectal cancer (CRC) predisposition.

METHODS: Peripheral blood samples of 510 study subjects (255 CRC patients, 255 controls)were collected. DNA was extracted and genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism. The association between polymorphic genotype and CRC predisposition was determined using the OR and 95%CI.

RESULTS: The frequency of the homozygous variant (Gln/Gln) genotype was significantly higher in cases compared with controls (16.0% *vs* 10.2%, $P = 0.049$). The Gln/Gln genotype of *XPC* showed a significantly higher association with the risk of CRC (OR = 1.884; 95%CI: 1.082-3.277; $P = 0.025$). In the case of allele frequencies, variant allele C was associated with a significantly increased risk of CRC (OR = 1.375; 95%CI: 1.050-1.802; $P = 0.020$). Moreover, the risk was markedly higher for those who were carriers of the Gln/Gln variant genotype and were also cigarette smokers (OR = 3.409; 95%CI: 1.061-10.949; $P = 0.032$).

CONCLUSION: The *XPC* Gln/Gln genotype alone and in combination with smoking increases the risk of CRC among Malaysians.

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Key words: DNA repair; Xeroderma pigmentosum group C Lys939Gln polymorphism; Cigarette smoking; Colorectal cancer; Susceptibility risk

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INTRODUCTION

Colorectal cancer (CRC) is a major public health problem worldwide, being the third most common cancer and the fourth most common cancer causing death[1]. In Malaysia, CRC has become the most common cancer among males and the second most common among females[2]. The development of CRC is a complex, multistep process involving interaction between environmental and genetic factors. Environmental factors such as dietary and lifestyle habits, smoking, alcohol consumption, and obesity interact with host's genetic factors, especially genetic variations, and may modulate the risk of CRC[3]. Genetic variations, such as single nucleotide polymorphisms (SNPs), may increase the sensitivity to environmental

carcinogens and may act as cancer predisposition factors. Environmentally sensitive genetic polymorphisms acting together with environmental factors are well documented candidates influencing cancer susceptibility.

DNA damage repair genes maintain the integrity of the genome against endogenous and exogenous factors. The xeroderma pigmentosum group C (*XPC*) gene is a DNA repair gene involved in the nucleotide excision repair (NER) mechanism which repairs bulky DNA lesions such as pyrimidine dimers, ultraviolet light-induced damage, photoproducts, intrastrand crosslinks, larger chemical adducts and other genotoxic agents[4]. Genetic variations in the *XPC* gene have been reported to modulate an individual's susceptibility to developing cancer[5,6]. The *XPC* Lys939Gln polymorphism, which leads to an amino acid change from lysine to glutamine at codon 939, is the most common SNP studied in the *XPC* gene and has been shown to be associated with increased risk of several cancers such as skin[7], breast[8] and bladder cancers[9,10].

However, there are only limited reports on the association of this polymorphism with CRC susceptibility. A case-control study was undertaken in order to investigate the association of this polymorphism alone and also in conjunction with cigarette smoking on the risk of CRC. This polymorphism is believed to alter the gene expression and modulate the DNA repair function of the protein product, as it is located at the coding sequence of the *XPC* gene. Thus, we hypothesized that *XPC* Lys939Gln polymorphism may have an effect on modulating the susceptibility to CRC, and cigarette smoking may further enhance the effect on CRC risk.

MATERIALS AND METHODS

Study subjects

The study was approved by the Research Review Board and Ethics Committee of Universiti Sains Malaysia, Kelantan and the Ministry of Health, Malaysia. For this hospital-based case control study, subjects were recruited from various hospitals in Malaysia, including Hospital Universiti Sains Malaysia, Hospital Raja Perempuan Zainab II and Hospital Sultanah Bahiyah, Kedah, Malaysia. Genotyping was carried out at the Human Genome Center, Universiti Sains Malaysia. Two hundred and fifty five sporadic CRC patients and 255 healthy normal controls were recruited as study subjects. Cases were histopathologically confirmed sporadic CRC patients, aged > 25 years, who did not have previous colon/rectal or other cancers. Cases with known (as indicated in the pathology reports) familial adenomatous polyposis, ulcerative colitis or Crohn's disease or any other previous malignancy were excluded. Controls were normal healthy individuals who were biologically unrelated to the study patients, aged > 25 years and had no history of cancer. Epidemiological data was collected from patients using a pre-structured questionnaire, which included sociodemographic status, physical status, dietary factors, occupation, tobacco/alcohol habits, previous illness, radiation exposure, *etc*.

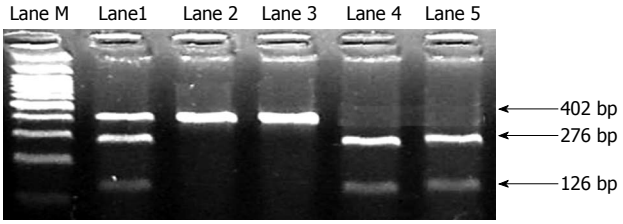

Figure 1 Gel picture showing the different categories of xeroderma pigmentosum group C Lys939Gln polymorphism genotype. Lane M: 100 bp DNA ladder; Lanes 2 and 3: Homozygous wildtype; Lane 1: Heterozygous; Lanes 4 and 5: Homozygous variant.

Genotyping

Genotyping of *XPC* Lys939Gln polymorphism was carried out using polymerase chain reaction (PCR)-restriction fragment length polymorphism. Briefly, PCR primers for *XPC* Lys939Gln (F: 5'-GGCTTCCTGGTATCTGATTACT-3'R: 5'-CTCAGTTTGCCTTCTCAGCA-3') were used to generate a 402 bp product containing the polymorphic site. The PCR reactions were carried out in a 25 μL volume of 1 × PCR Buffer, 2.0 mmol/L of $MgCl_2$, 0.5 mmol/L dNTPs, 0.4 mmol/L of each primers and 1 U of AmpliTaq Gold Polymerase with a denaturation of 94 ℃ for 5 min, followed by 35 cycles at 94 ℃ for 30 s, 57 ℃ for 30 s, 72 ℃ for 30 s and finally 5 min at 72 ℃. Following amplification, the PCR products were digested using *PvuII* restriction enzyme for 1 h at 37 ℃ and electrophoresed on 2% agarose gel. The homozygous wild-type genotype was identified by a single band at 402 bp level, the heterozygous genotype by 3 bands at 402, 276 and 126 bp levels and the homozygous variant by 2 bands at 276 and 126 bp levels (Figure 1).

Statistical analysis

The sample size was calculated with power and sample size (PS) software version 2.1.31 using the uncorrected χ^2 test with 80% power and 95%CI. The difference in distribution of genotypes, gender and age between cases and controls were assessed using the χ^2 test. The ORs and 95%CI were calculated using binary logistic regression to evaluate the risk association. All statistical tests were two-sided, and statistical significance was determined as $P < 0.05$. SPSS v.18 (SPSS Inc., Chicago, IL, United States) was utilized for statistical analysis.

RESULTS

The demographic characteristics of the study subjects are shown in Table 1. This case-control study recruited 510 study subjects, including 255 histopathologically confirmed sporadic CRC patients and 255 healthy normal controls. Of the 255 CRC patients, 139 (54.5%) were male and 116 (45.5%) were female. Of the 255 normal controls, 115 (45.1%) were male and 140 (54.9%) were female. The ages of the sporadic CRC patients ranged from 27 to 93 years (mean age: 53.17 ± 7.07 years) and of the healthy normal controls ranged from 26 to 84

Table 1 Demographic characteristics of the study population *n* (%)

Variable	Cases (*n* = 255)	Controls (*n* = 255)	*P*-value
Gender			
Male	139 (54.5)	115 (45.1)	0.034[1]
Female	116 (45.5)	140 (54.9)	
Mean age (yr)	53.17 ± 7.07	46.47 ± 12.02	0.086
Cigarette smoking			
Yes	110	48	< 0.001[1]
No	145	207	

[1]Statistically significant.

Table 2 Genotype and allele frequencies of the xeroderma pigmentosum group CLys939Gln polymorphism in study subjects *n* (%)

	Cases (*n* = 255)	Controls (*n* = 255)	*P*-value
Genotype			
Homozygous wildtype (AA)	108 (42.4)	129 (50.6)	0.062
Heterozygous (AC)	106 (41.6)	100 (39.2)	0.588
Homozygous variant (CC)	41 (16.0)	26 (10.2)	0.049[1]
Allele			
A	322 (63.1)	358 (70.0)	0.294
C	188 (36.9)	152 (30.0)	

[1]Statistically significant.

years (mean age: 46.47 ± 12.02 years).

The frequencies of the homozygous wildtype (Lys/Lys), heterozygous (Lys/Gln) and homozygous variant (Gln/Gln) genotypes were 108 (42.4%), 106 (41.6%) and 41 (16.0%), respectively, among CRC cases. In controls, the frequencies were 129 (50.6%) for homozygous wildtype, 100 (39.2%) for heterozygous and 26 (10.2%) for homozygous variant genotypes. The frequency of the homozygous variant (CC) genotype was significantly higher in cases compared with the controls ($P = 0.049$). Table 2 shows the genotype and allele frequencies of *XPC* Lys939Gln polymorphism in cases and controls.

Binary logistic regression analysis was performed in order to find the risk association. Table 3 shows the association between the *XPC* Lys939Gln polymorphism and risk of CRC. It can be clearly seen that the homozygous variant genotype was significantly associated with increased risk of CRC (OR = 1.884; 95%CI: 1.082-3.277; $P = 0.025$). The variant allele C was found to be significantly associated with increased risk of CRC (OR = 1.375; 95%CI: 1.050-1.802; $P = 0.020$). Furthermore, the study subjects were stratified into smokers and non-smokers and the risk association was evaluated. Results showed that, for those with the homozygous variant genotype (CC) and who were smokers, the risk was significantly increased (OR = 3.409; 95%CI: 1.061-10.949; $P = 0.032$). In the non-smoking group, no significant association was observed between the *XPC* Lys939Gln polymorphism and the risk of CRC.

Table 3 Xeroderma pigmentosum group C Lys939Gln polymorphism, smoking and colorectal cancer susceptibility risk

	Cases	Controls	OR (95%CI)	*P*-value
Genotype				
Lys/Lys (AA)	108	129	1 (Reference)[1]	
Lys/Gln (AC)	106	100	1.266 (0.871–1.841)	0.216
Gln/Gln (CC)	41	26	1.884 (1.082–3.277)	0.025[2]
Allele				
A	322	358	1 (Reference)[1]	-
C	188	152	1.375 (1.050–1.802)	0.020[2]
Non-smoker				
AA	64	104	1 (Reference)[1]	
AC	64	81	1.284 (0.817–2.018)	0.278
CC	17	22	1.256 (0.620–2.542)	0.526
Smoker				
AA	44	25	1 (Reference)[1]	
AC	42	19	1.256 (0.605–2.609)	0.541
CC	24	4	3.409 (1.061–10.949)	0.032[2]

[1]Homozygous wildtype genotype served as the reference category; [2]Statistically significant.

DISCUSSION

To date, a variety of chemical carcinogens have been identified to cause DNA damage in humans. DNA damage repair genes are responsible for maintaining the integrity of the human genome through different pathways, locations and types of damage by base excision repair, NER, double strand break and mismatch repair pathways[4]. Genes involved in the NER pathway, such as *XPC*, commonly repair lesions induced by numerous exogenous agents such as those derived from food and smoking, including 2-amino-1-methyl-6-phenylimidazo-[4,5-β]-pyridine and benzo-[α]-pyrenediol-epoxide[11].

The *XPC* gene, located on chromosome 3p25, contains 16 exons and 15 introns and encodes a 940 amino acid protein[12]. Several polymorphic variants in the *XPC* gene have been identified and *XPC* Lys939Gln is one of the three most common SNPs. This case-control study investigated the genotype and allele frequencies of the *XPC* Lys939Gln polymorphism, and the risk association with sporadic CRC susceptibility. Results from the present study showed that the frequency of the *XPC* homozygous variant genotype was significantly higher in cases compared with controls (16.0% *vs* 10.2%, $P = 0.049$). On evaluation of the risk association, the homozygous variant genotype (CC) was associated with significantly increased risk of CRC (OR = 3.409; $P = 0.032$). This result conforms with a study by Yasuda *et al*[13], which demonstrated that a single amino acid alteration could be sufficient to compromise *XPC* function, thereby enhancing the risk of CRC development. Our results are in agreement with a few other reports by Qiu *et al*[5], Zhang *et al*[6] and Gil *et al*[14] which revealed that *XPC* is one the most important genes modulating an individual's risk of developing sporadic cancer.

Carcinogens consumed through dietary and lifestyle habits as well as from the environment play a major role

in damaging DNA. Cigarette smoking is one of the lifestyle factors that play an important role in the exposure of an individual to the carcinogens present in cigarette smoke. Carcinogens contained in tobacco smoke, such as polycyclic aromatic hydrocarbons, heterocyclic amines, nitrosamines and aromatic amines, are harmful to the human colon and rectum. Cigarette smoking has been reported to lead to the formation of DNA adducts and cause damage to the DNA in the human colon[15,16]. All these carcinogens can reach the colorectal mucosa, through direct ingestion or via the circulatory system, and has been demonstrated to induce bulky adducts in crypt cells and contribute to the formation of mutations in the colon[17,18]. In the present study, a combination of smoking and a variant genotype of *XPC* enhanced the risk of CRC 3-fold, indicating that smoking further enhanced the risk of CRC. Although non-smokers showed an OR of 1.256, this was not statistically significant.

The *XPC* gene encodes an important protein involved in the recognition of DNA damage in the NER pathway. This protein binds to HR23B protein to form XPC-HR23B that recognizes a DNA lesion and repairs the DNA damage along with other DNA repair proteins[19,20]. Polymorphism in the coding sequence of the *XPC* gene has been demonstrated to alter the gene expression and thereby modulate the DNA repair function[21]. The *XPC* Lys939Gln polymorphism is located in the coding sequence of the *XPC* gene. The nucleotide change from A to C leads to an amino acid change from lysine to glutamine in the coding sequence of the *XPC* gene and has been reported to lead to reduced repair capacity. This genetic variation has also been reported to result in reduced specificity of this gene in recognition and repair of the DNA damage as well as in protein expression, thus allowing more somatic DNA mutations or alterations to occur[21,22].

The XPC protein plays a crucial role in repairing the DNA damage caused by tobacco smoke. Individuals with the *XPC* variant genotype may possess deficient DNA repair capability. Accordingly, the XPC protein product may be less efficient in repairing the DNA lesions induced by tobacco smoke, and thereby could enhance the susceptibility, favoring the development of CRC.

Previous studies focusing on the role of the *XPC* variant in the modulation of an individual's risk of developing CRC are scarce, and reported results are inconsistent. Berndt *et al*[22], in an American population, found an OR of 1.19 for the variant genotype's association with risk of CRC, but the result was statistically insignificant (P = 0.50). In contrast, a study conducted by Hansen *et al*[23], in a Polish population, observed no significant risk association between this polymorphism and CRC susceptibility. However, when the variant genotype in conjunction with red meat consumption was evaluated, the risk was reported to be significantly higher (OR = 3.7) in those who carried the homozygous variant (CC) genotype and consumed 100 g red meat per day.

A recent study by Wu *et al*[24] on 421 CRC patients and 845 controls, showed that the homozygous variant (CC) genotype was significantly associated with higher risk of CRC in a Chinese population (OR =1.5; 95%CI: 1.0-2.2; P = 0.035). Moreover, combination (AC + CC) genotypes in the homozygous wildtype model also showed a significant association with risk of CRC (OR = 1.3; 95%CI: 1.0-1.7; P = 0.039). When the study subjects were stratified into tea drinkers and non-tea drinkers, the results showed that individuals who were non-tea drinkers and carried combination (AC + CC) genotypes, had increased risk of CRC (OR = 3.0; 95%CI: 1.9-4.7; P < 0.001).

Apart from CRC, *XPC* polymorphism was reported to be associated with the risk of other cancers, such as head and neck[25], lung[10], breast[8] and bladder[26]. In addition, abnormal expression of the *XPC* gene has been shown in hepatocarcinogenesis[27]. The variant allele of the *XPC* gene is associated with lower DNA repair capacity (DRC). Several studies showed that the Lys939Gln polymorphism had an interaction with other SNPs located in the same gene, such as Ala499Val, PAT (-/+) and IVS11 C/A polymorphisms. According to Khan *et al*[28], the *XPC* Lys939Gln polymorphism is in linkage disequilibrium with the *XPC* PAT polymorphism as well as the C/A polymorphism at 25 positions of intron 11 of *XPC*. This causes exon 12 to be skipped and deleted, resulting in the loss of *XPC* cDNA function, thus reducing the DNA repair activity.

Researchers have shown that carriers of the C variant allele of the *XPC* Lys939Gln polymorphism exhibited significantly more DNA damage induced by BPDE[21,29,30]. Moreover, combinations of haplotype C + C (Lys939Gln, PAT +, Ala499Val) have been reported to cause poor DRC, thus increasing the risk of CRC. In contrast, haplotype T-A (Ala499Val-PAT-Lys939Gln) has been demonstrated to be associated with the least DNA damage[21,24]. Also, another study has shown that accumulation or the presence of alleles in the *XPC* gene associated with increased risk might reduce DRC and thus increase CRC susceptibility[31].

The difference in results on the risk association between the present study and other previous studies might be explained by differences in genetic background of study subjects groups or populations, and also differences in exposure to environmental and lifestyle factors. Small sample sizes and/or inadequate control for certain confounder factors such as gender and age might also have contributed to the differing results and lack of association.

From the present study, it is reasonable to suggest that the *XPC* gene, especially the *XPC* variant genotype, may modulate the DRC of the host cell and thus play an important role in sporadic colorectal carcinogenesis. The risk could be much higher in individuals who possess the variant genotype and who are also cigarette smokers. The results also highlight the potential role of the NER pathway (especially of *XPC*) in modulation of an individual's risk of sporadic CRC. Further studies to explore the interaction of *XPC* Lys939Gln with other SNPs of

the *XPC* gene and with other genes involved in DNA repair pathways, either singly or in combination, and to examine the correlation with environmental factors such as alcohol consumption and dietary habits, as well as clinicopathological characteristics, would be beneficial in deriving more accurate risk predictive markers.

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COMMENTS

Background

Colorectal cancer (CRC) which is the most common cancer among males and the second most common among females in Malaysia, has become a major public problem. Environmental factors, such as dietary carcinogens and tobacco smoke, interacting with the host's genetic factors may modulate the risk of CRC. However, the genetic predisposition factors associated with colorectal carcinogenesis remain largely undetermined. Identification of the host's genetic predisposition factors may help in understanding the carcinogenic process. Thus, it was of interest to explore the contribution of single nucleotide polymorphisms (SNPs) in a DNA repair gene, alone and in combination with tobacco smoke, as a predisposition factor for CRC susceptibility.

Research frontiers

Exposure to mutagens and carcinogens through diet, tobacco smoke,*etc* can cause varying degrees of DNA damage and can cause mutations in humans if left unrepaired. The xeroderma pigmentosum group C (*XPC*) gene is a DNA repair gene involved in the nucleotide excision repair (NER) pathway. The SNP, Lys939Gln, located at the coding region of the *XPC* gene has been associated with lower DNA repair capacity and has been shown to be associated with increased risk of cancers of the skin, breast and bladder. This study was designed to determine the frequencies and influence of the *XPC* Lys939Gln polymorphic genotype, alone and in combination with smoking, on sporadic CRC susceptibility risk in a Malaysian population.

Innovations and breakthroughs

Only limited reports are available on the association of *XPC* Lys939Gln polymorphism with CRC susceptibility risk. To the best of our knowledge, this is the first report of an association between the genetic variant of *XPC* with CRC susceptibility risk in the Malaysian population. The observation that genetic variation of the *XPC* gene influences susceptibility to CRC implies the importance of the NER pathway in modulation of an individual's risk of CRC. This study provides support for the hypothesis that genetic variation in the *XPC* gene, acting together with environmental factors, contributes to CRC development and may be considered as a candidate marker for CRC predisposition risk in the Malaysian population.

Applications

The present study observed that the genetic variation Lys939Gln of the *XPC* gene influences the risk of CRC in the Malaysian population. Further SNP mapping studies utilizing high throughout genotyping methods could facilitate the analysis of multiple polymorphisms within DNA repair genes. Genotyping of Malaysian CRC patients for polymorphism(s) in DNA repair genes will help in understanding the specific polymorphism(s), which act as predisposing genotype(s) in CRC susceptibility. In future, the study can be extended to a population level, to identify individuals with high risk predisposition genotypes. Appropriate surveillance programs could then be developed in order to reduce the morbidity and mortality of CRC at the population level.

Peer review

This case-control study investigated the association between SNPs and cigarette smoking on sporadic CRC susceptibility risk in Malaysian population. The genotype and allele frequencies of *XPC* Lys939Gln were investigated in 255 CRC patients and 255 healthy controls. Homozygous variant (CC) genotype frequency was found to be significantly higher in CRC cases compared to controls. When the risk association was evaluated singly, the homozygous variant *XPC* CC genotype was associated with an increased risk of CRC susceptibility. Moreover, when the risk was evaluated after stratifying the study subjects into smokers and non-smokers, the combination of smoking habits and variant genotype of *XPC* was found to enhance the CRC susceptibility risk to 3-fold times higher. These results prompt us to suggest that genetic variation Lys939Gln in *XPC* gene might modify the effect of smoking and contribute to sporadic colorectal cancer etiology.

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BRIEF ARTICLE

Role of international criteria in the diagnosis of autoimmune hepatitis

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Abstract

AIM: To study the clinical and laboratory characteristics of autoimmune hepatitis (AIH), and compare them with International Autoimmune Hepatitis Group (IAHG) criteria.

METHODS: Sixty consecutive patients with AIH attended the University Clinic at Tabriz University of Medical Sciences, Iran for a 12 mo period and were assessed in a case series study. Serological and biochemical evaluations were carried out in all patients. Autoantibodies, such as antinuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-liver-kidney microsomal antibody (ALKM-1) type 1, and perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA) were evaluated in these patients. A liver biopsy was performed after diagnosis of the disease. Patients were evaluated in terms of their signs and symptoms, and laboratory results and the degree to which they corresponded with the diagnostic criteria of IAHG. In this study, both a comprehensive diagnostic scoring system and a simplified diagnostic scoring system were employed for AIH.

RESULTS: Sixty patients, 20 male, 40 female, mean age 39.45 ± 17.50 years, participated in the study. Treatment began immediately after enrolment into the study. The percent distribution of the study population into definite and probable did not change after the treatment. The most common symptoms in descending order were fatigue (100%), icter (66.7%), abdominal discomfort (33.3%), abdominal distension (28.3%), dark urine (23.3%), edema (23.3%), hematemesis (20.0%), pruritus (20.0%), melena (11.7%) and pale stool (10.0%). At the physical examination, splenomegaly, ascites, hepatomegaly, epigastric tenderness and an abdominal mass were found in 50.0%, 16.7%, 13.3%, 5.0% and 3.3% of patients, respectively. Hypergammaglobulinemia was detected in 95.0% of cases. ALKM-1, P-ANCA, ANA and ASMA were positive in 71.4%, 66.7%, 42.4% and 19.4% of cases, respectively. Portal hypertensive gastropathy (45.0%), esophageal varices (41.7%) and cirrhosis (40.0%) were the most prevalent complications of AIH, and there was no evidence of primary sclerosing cholangitis, ulcerative colitis and overlap syndrome in these patients. According to IAHG criteria, 80.0% of cases had a definite diagnosis, 15.0% had a probable diagnosis and 5.0% had no AIH. The percent distribution of the study population into definite, probable and no AIH did not change after using the simplified diagnostic scoring system for AIH.

CONCLUSION: This research showed that the majority of cases in our study were appropriately diagnosed according to the IAHG criteria and simplified scoring system. Thus, these criteria are very useful.

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Key words: Autoimmune hepatitis; International criteria; Diagnosis; Clinical; Paraclinical

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INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic inflammation of the liver, the cause of which is unknown. It is characterized by the presence of interface hepatitis on histological examination, hypergammaglobulinemia, and autoantibodies[1]. Autoimmune hepatitis occurs predominantly in women and affects all ages[2]. Autoimmune hepatitis afflicts 100000 to 200000 persons in the United States[3], and accounts for 2.6% of transplant recipients in Europe[4] and 5.9% in the United States[5]. Among Northern European Caucasians, the mean annual incidence of AIH is 1.9 per 100000 population, and its point prevalence is 16.9 per 100000 population[6]. Three types of AIH have been proposed based on immunoserologic markers[7]. The International Autoimmune Hepatitis Group (IAHG) devised and subsequently revised scoring systems to aid in the diagnosis of AIH[8,9]. The revised 1999 criteria evaluated up to 12 patient variables to derive a score which identified individuals as "not AIH", "probable AIH" or "definite AIH"[8]. Despite a high degree of sensitivity and specificity, these criteria have proven cumbersome in day-to-day clinical practice. Subsequently, the IAHG published simplified diagnostic criteria, evaluating just four parameters[10]. This study was designed to determine a standard and reliable method for early diagnosis and close follow-up by using the IAHG simplified diagnostic criteria and comparing clinical and laboratory characteristics in Iranian AIH patients.

MATERIALS AND METHODS

All patients who had been diagnosed with autoimmune hepatitis and had been referred to the outpatient clinic of Tabriz University of Medical Sciences from 2010 to 2011 were evaluated for clinical and laboratory parameters and compared with the diagnostic criteria of IAHG. Patient evaluation started by recording their medical history, and performing a physical examination and complete blood count. Serological and biochemical evaluation included aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, serum γ-globulin, serum albumin, total and direct bilirubin, erythrocyte sedimentation rate, prothrombin time, serum creatinine, triglyceride, total cholesterol and fasting blood sugar. The researchers also evaluated the autoantibodies such as antinuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-liver-kidney microsomal antibody (ALKM-1) type 1 and perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA) by indirect immunofluorescence. A titer of $\geqslant$ 1:40 was considered to be positive. Chronic viral hepatitis, Wilson's disease and hemochromatosis were also assessed using hepatitis B core antibody, hepatitis B surface antibody, hepatitis C antibody, serum ceruloplasmin, urine copper, serum iron and total iron-binding capacity. Hepatitis B surface antigen was detected by enzyme-linked immunosorbent assay (Stat Fax Awareness Technology Inc., Palm City, FL, United States) and hepatitis C virus (HCV) antibody was analyzed using a third generation enzyme linked immunosorbent assay test (Ortho-Clinical Diagnostics, Amersham, United Kingdom).

A percutaneous liver biopsy was taken from all patients for histology after diagnosis of the disease. Specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin, Masson's trichrome, and reticulin. All specimens were evaluated by a single pathologist. Liver biopsies were adequate if there were at least 6 portal tracts per high-power field. A modified Hepatitis Activity Index was used to score specimens, in which necroinflammation was graded from 0 to 18 and fibrosis from 0 to 6[11].

An abdominal ultrasound examination was performed in all patients, and liver size and echogenicity, splenomegaly, gallstones and ascites were assessed. The exclusion criteria were having viral (hepatitis B and C), metabolic (Wilson's disease, hemochromatosis), or drug-induced liver disease or overlap syndrome. Continuous variables are expressed as mean ± SD. Statistical analysis was carried out using SPSS, version 16.0 (SPSS Inc., Chicago, IL, United States). The study protocol was approved by the Ethics Committee of the Liver and Gastrointestinal Diseases Research Center of Tabriz University of Medical Sciences, and informed consent was procured from all patients before enrolment in the study.

RESULTS

Sixty patients with AIH were evaluated, of whom 40 (66.7%) were female. The mean age was 39.45 ± 17.50 years (range, 19-75 years). Twelve patients had a familial history of liver disease (20.0%) and 8 patients had a familial history of AIH (13.3%). Four patients (6.7%) were diagnosed with other simultaneous autoimmune diseases. None of the patients had a history of hepatotoxic drug use. Patient characteristics are described in Table 1.

The most common symptoms in descending order were fatigue (100%), icterus (66.7%), abdominal discomfort (33.3%), abdominal distension (28.3%), dark urine (23.3%), edema (23.3%), hematemesis (20.0%), pruritus (20.0%), melena (11.7%) and pale stools (10.0%). At the time of physical examination, splenomegaly, ascites, hepatomegaly, epigastric tenderness and abdominal mass were found in 50.0%, 16.7%, 13.3%, 5.0% and 3.3% of the patients, respectively. Portal hypertensive gastropathy (45.0%), esophageal varices (41.7%) and hepatic cirrhosis (40.0%) were the most common complications in the patients.

The proportion of patients seropositive for ALKM1, P-ANCA, ANA and ASMA patients was 71.4%, 66.7%,

Table 1 Characteristics of patients with autoimmune hepatitis

Parameter	*n* (%)
Female	40 (66.7)
Male	20 (33.3)
Single	22 (36.7)
Married	38 (63.3)
Educated	46 (76.7)
Uneducated	14 (23.3)
Alcohol intake	3 (5)
Smoking	11 (18.3)
History of blood transfusion	17 (28.3)
History of hospitalization	48 (80)

Table 2 Serological, biochemical and histologic findings in patients with autoimmune hepatitis

Parameter	Result	Range
AST, IU/L	127.8 ± 108.6	17-812
ALT, IU/L	146.0 ± 98.4	14-916
Total bilirubin, mg/dL	2.7 ± 2.2	0.5-8.6
Direct bilirubin, mg/dL	1.3 ± 1.2	0.05-4.3
Alkaline phosphatase, IU/L	499.9 ± 386.2	71-997
White blood cell, No./mm^3	6052.4 ± 2461.5	800-13500
Hemoglobin, g/dL	12.1 ± 3.0	7-17
ESR	40.0 ± 28.7	3-95
Platelet count (× 1000), No./mm^3	139.2 ± 91.4	36.4-464
Prothrombin time	15.8 ± 3.3	12.5-31
Creatinine, mg/dL	0.8 ± 0.3	0.5-2.1
FBS, mg/dL	106.9 ± 48.6	68-302
Triglyceride, mg/dL	163.2 ± 109.5	50-506
Total cholesterol, mg/dL	208.1 ± 106.3	47-472
Albumin, g/dL	3.8 ± 0.7	2.1-5.1
Grade	5.5 ± 2.7	
Stage	3.2 ± 1.5	

All values are mean ± SD. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ESR: Erythrocyte sedimentation rate; FBS: Fasting blood sugar.

42.4% and 19.4% respectively. Simultaneous seropositivity for ASMA/ANA occurred in 6.7%, ANA/P-ANCA in 6.7%, ANA/ALKM1 in 5.0%, and ALKM1/ASMA and/or ANA in 3.3%. Hepatitis C virus antibody was assessed in all patients, and all were negative. There was no evidence of primary sclerosing cholangitis, ulcerative colitis or overlap syndrome in these patients.

Liver biopsy and histological assays were performed in all the patients, and all had interface hepatitis. Forty-five cases of pathology reports were descriptive and the rest showed autoimmune hepatitis. The researchers found portal fibrotic expansion (stage 1-2) in 15 patients (33.3%), bridging fibrosis (stage 3-4) in 17 patients (37.8%) and cirrhosis (stage 5-6) in 13 patients (28.8%).

Blood proteins (electrophoresis analysis) were assessed in patients whose mean level of α1 protein was 3.0 ± 1.6 g/dL (0-7.1 g/dL). The mean level of α2 protein was 10.0 ± 3.2 g/dL (0.3-16.6 g/dL). The mean level of β protein was 11.4 ± 4.4 g/dL (0.5-17.0 g/dL). The mean level of γ protein was 25.8 ± 11.4 g/dL (3.7-44.1 g/dL). Hypoalbuminemia was found in 20 (33.3%) patients. Further serological and biochemical data are shown in Table 2.

Table 3 Autoimmune hepatitis diagnosis using simplified score and revised International Autoimmune Hepatitis Group criteria

AIH score	Probable AIH	Definite AIH	No AIH
Revised IAHG			
Pre-treatment	17.7 ± 1.7	12.8 ± 1.4	7.7 ± 1.5
Post-treatment	19.7 ± 1.4	14.8 ± 1.4	9.7 ± 1.5
Simplified	7.5 ± 0.5	5.6 ± 0.5	3.3 ± 0.6

Data are mean scores ± SD. AIH: Autoimmune hepatitis; IAHG: International Autoimmune Hepatitis Group.

Liver and bile duct ultrasonic imaging was performed in all patients and increased echogenicity in liver was found in 36 (60.0%). Splenomegaly was found in 32 patients (53.3%). Increased liver size (hepatomegaly) was found in 12 patients (20.0%), decreased liver size in 20 (33.3%) and normal liver size in 28 (46.7%). A gallstone was found in 11 patients (18.3%) a dilated bile duct in 9 (15.0%), and ascites in 13 (21.7%).

There was a good outcome during the 1-year follow-up in 58 patients (96.7%), one patient (1.7%) had no response to treatment and one patient with complications died while the research was being conducted. The 1-year mortality rate was 1.7%.

According to the revised IAHG criteria, 80.0% of cases had a definite diagnosis of AIH, 15.0% of cases had a probable diagnosis and 5.0% of cases had no AIH. Using the simplified diagnostic scoring system for AIH, the percent distribution of the study population into definite, probable and no AIH did not change. The mean scores and standard deviations for both the revised IAHG and simplified scoring criteria are presented in Table 3.

DISCUSSION

The researchers surveyed 60 patients with AIH. The most common symptoms were fatigue, icterus, abdominal discomfort, abdominal distension, dark urine, edema, hematemesis, pruritus, melena and pale stools. At the physical examination, splenomegaly, ascites, hepatomegaly, epigastric tenderness and abdominal mass were discerned in 50.0%, 16.7%, 13.3%, 5.0% and 3.3% of the patients, respectively.

Koay *et al*[12] had performed a similar study in Taiwan, and the most common clinical findings of AIH were fatigue, icterus and loss of appetite. Another study conducted by Choudhuri *et al*[13] in India reported icterus (55.2%), edema (44.7%), fatigue (44.7%), encephalopathy (23.6%), pruritus (23.6%), abdominal pain (23.6%), fever (21.0%), arthritis (18.4%), hepatomegaly (44.7%), splenomegaly (34.2%) and ascites (34.2%) in their clinical findings. In a study by Gupta *et al*[14] in India (2001), the most common manifestations were fatigue, icterus and loss of appetite.

Variable findings can be seen in the studies reported above. Nevertheless, there is a similarity between the

general findings of the present study and those studies. The characteristic laboratory findings in the patients in the present study were an increase in bilirubin and abnormalities in liver enzyme levels. The pathologic found interface hepatitis in all patients, and 71.4%, 66.7%, 42.4% and 19.4% seropositivity for ALKM1, P-ANCA, ANA and ASMA, respectively.

Koay *et al*[12] reported abnormalities in liver tests and increased bilirubin. Patients were 98.0% positive for ANA. Zhao *et al*[15] in China reported interface hepatitis in all their AIH cases. In a study by Choudhuri *et al*[13], reported positivity for ANA in 39.4%, ASMA in 63.1% and P-ANCA in 2.6%. Johnson *et al*[9] reported ANA or ASMA in 70.0%-80.0% and ALKM1 in 3.0%-4.0%. In a study by Nezu *et al*[16] in Japan, 34.0% of patients were positive for ANA. In another study in Japan by Omagari *et al*[17], 34.0% of patients were positive for ANA. In another study by Terjung *et al*[18] in 175 patients, 81.0% were positive for P-ANCA. Pavić *et al*[19] in Serbia had reported ANA seropositivity in 15.0%-60.0%, ASMA in 34.0%-60.0% and ALKM1 in 0-6.0%. In a study by Adams *et al*[20] in the United States, 20.0% of patients were positive for ANA and 3.0% for ASMA.

In summary, the range of antibody positivity in the above-mentioned studies were: ANA 15.0%-98.0%, ASMA 3.0%-80.0%, ALKM1 0%-6.0% and P-ANCA 2.6%-81.0%. The ranges in the present study are mostly in the reported ranges. Although technical differences in measurement of antibodies in different centers can produce some variability in the results, these wide differences may be a sign of racial differences in patients with AIH. This issue requires more controlled studies. Also, the present study had a small sample of patients and larger studies are required in future to examine the applicability of antibody measurements.

In the present study, 95.0% of patients were diagnosed using the diagnostic criteria of the IAHG. Several studies which had been conducted around the world that insisted on using these criteria, including Heurgué *et al*[21] in Italy, Koay *et al*[12] in Taiwan (China), Lee *et al*[22] in Southern Korea, Michalska *et al*[23] in Poland, Primo *et al*[24] in Spain, Zhao *et al*[15] in China, McFarlane *et al*[25] in England and Yatsuji *et al*[26] in Japan.

In conclusion, this research showed that the majority of cases in the present study were diagnosed according to the criteria of IAHG and the simplified scoring system. There were some deficiencies in the assessment of autoantibodies; therefore, recommendations are made for controlled studies to diagnose the probable causes of these deficiencies. The results of this study conform with reports in the literature. Further studies should be carried out in similar centers.

COMMENTS

Background

Autoimmune hepatitis (AIH) is an unresolving inflammation of liver, the cause of which is unknown. It is characterized by the presence of interface hepatitis on histological examination, hypergammaglobulinemia, and autoantibodies.

Research frontiers

Despite a high degree of sensitivity and specificity, the diagnostic criteria have proven cumbersome in day-to-day clinical practice. Subsequently, the International Autoimmune Hepatitis Group (IAHG) have published simplified diagnostic criteria, evaluating just four parameters.

Innovations and breakthroughs

This study was designed to determine a standard and reliable method for early diagnosis and close follow-up by using IAHG criteria and simplified diagnostic criteria and comparing clinical and laboratory characteristics in Iranian patients.

Applications

This research showed that the majority of cases in the present study were diagnosed according to the criteria of IAHG and simplified scoring system. There were some deficiencies in autoantibodies assessment; therefore, recommendations are made for controlled studies to diagnose the probable causes of these deficiencies.

Peer review

The authors collected data for one of the largest AIH cohort in the Middle East and compared laboratory and histologic findings with the international AIH Score. Using the score they found a definite AIH in 80% of their patients and a probable AIH in 15%. So the authors showed that the score is useful and can be used in patients of the Middle East without limitations. Altogether the achievement of the study is to collect such a cohort in a single center. The study was well performed.

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BRIEF ARTICLE

Focal autoimmune pancreatitis: Radiological characteristics help to distinguish from pancreatic cancer

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Abstract

AIM: To identify the radiological characteristics of focal autoimmune pancreatitis (f-AIP) useful for differentiation from pancreatic cancer (PC).

METHODS: Magnetic resonance imaging (MRI) and triple-phase computed tomography (CT) scans of 79 patients (19 with f-AIP, 30 with PC, and 30 with a normal pancreas) were evaluated retrospectively. A radiologist measured the CT attenuation of the pancreatic parenchyma, the f-AIP and PC lesions in triple phases. The mean CT attenuation values of the f-AIP lesions were compared with those of PC, and the mean CT attenuation values of pancreatic parenchyma in the three groups were compared. The diagnostic performance of CT attenuation changes from arterial phase to hepatic phase in the differentiation between f-AIP and PC was evaluated using receiver operating characteristic (ROC) curve analysis. We also investigated the incidence of previously reported radiological findings for differentiation between f-AIP and PC.

RESULTS: The mean CT attenuation values of f-AIP lesions in enhanced phases were significantly higher than those of PC (arterial phase: 60 ± 7 *vs* 48 ± 10, $P < 0.05$; pancreatic phase: 85 ± 6 *vs* 63 ± 15, $P < 0.05$; hepatic phase: 95 ± 7 *vs* 63 ± 13, $P < 0.05$). The mean CT attenuation values of f-AIP lesions were significantly lower those of uninvolved pancreas and normal pancreas in the arterial and pancreatic phase of CT ($P < 0.001$, $P < 0.001$), with no significant difference at the hepatic phase or unenhanced scanning ($P = 0.4$, $P = 0.1$). When the attenuation value increase was equal or more than 28 HU this was considered diagnostic for f-AIP, and a sensitivity of 87.5%, specificity of 100% and an area under the ROC curve of 0.974 (95%CI: 0.928-1.021) were achieved. Five findings were more frequently observed in f-AIP patients: (1) sausage-shaped enlargement; (2) delayed homogeneous enhancement; (3) hypoattenuating capsule-like rim; (4) irregular narrowing of the main pancreatic duct (MPD) and/or stricture of the common bile duct (CBD); and (5) MPD upstream dilation ⩽ 5 mm.

CONCLUSION: Analysis of a combination of CT and MRI findings could improve the diagnostic accuracy of differentiating f-AIP from PC.

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Key words: Focal autoimmune pancreatitis; Pancreatic cancer; Computer tomography; Magnetic resonance imaging; Magnetic resonance cholangiopancreatography

Core tip: At present, focal autoimmune pancreatitis (f-

AIP) is still very difficult to differentiate from pancreatic cancer (PC). In this study, we compared the incidence of radiological features, investigated the differences in the triple-phase enhancement pattern of f-AIP and PC, and found that the combination analysis contributed to improve the diagnostic accuracy of f-AIP thus avoiding unnecessary surgery.

Sun GF, Zuo CJ, Shao CW, Wang JH, Zhang J. Focal autoimmune pancreatitis: Radiological characteristics help to distinguish from pancreatic cancer. *World J Gastroenterol* 2013; 19(23): 3634-3641 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3634.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3634

INTRODUCTION

Autoimmune pancreatitis (AIP) is a rare form of immune-mediated chronic pancreatitis (CP) due to an autoimmune mechanism and is characterized by a marked infiltration of lymphocytes and plasma cells in pancreatic tissue (lymphoplasmacytic sclerosing pancreatitis) and was first described in 1961[1-5]. It can be classified into two radiological types: the diffuse form (the most frequent, 70% of cases) and the focal form (30% of cases), which is characterized by focal swelling of the pancreas, localized narrowing of the main pancreatic duct (MPD) with an irregular wall on imaging modalities[6-8]. Focal AIP (f-AIP) can be due to a mass formation or swollen pancreas located in one or two segments of the gland. AIP has a variety of manifestations, obstructive jaundice occurs in 76% and weight loss in 35% of patients, usually in combination with pancreatic enlargement, especially with focal pancreatic enlargement on imaging, making it very difficult to differentiate from pancreatic cancer[7,9,10].

Treatment for AIP and pancreatic cancer (PC) are completely different. Autoimmune pancreatitis is a benign disease and steroid therapy can rapidly resolve symptoms; for PC, however, surgical resection is preferred, and if necessary, PC patients might receive combined radiotherapy or chemotherapy. However, about 3%-5% of patients undergoing pancreatic resection for presumed PC in fact have AIP[11]. Kamisawa *et al*[12] reported that 7 of 37 (18.9%) AIP patients had surgery because they were misdiagnosed as having PC or bile duct cancer. In particular, it is very difficult to differentiate between f-AIP and PC. Chang *et al*[13] reported that 8 of 26 (31.8%) AIP patients had f-AIP who were frequently treated with surgery because differentiating f-AIP from PC was so difficult. It was reported that approximately 2.2%-35.2% of f-AIP patients had undergone surgery due to a presumed diagnosis of PC[12,14-16].

Here, we report the radiological features of 19 cases of f-AIP presenting as PC to: (1) increase the awareness and recognition of f-AIP; and (2) improve the diagnostic accuracy and avoid unnecessary surgery due to misdiagnosis.

MATERIALS AND METHODS

Patient sample

This study was approved by the hospital ethical committees. Consecutive patients with focal autoimmune pancreatitis (f-AIP, n = 19) were recruited between November 2009 and October 2012. Patients with AIP were diagnosed according to the Asian Diagnostic Criteria for Autoimmune Pancreatitis (2008)[17]. A diagnosis of AIP was established when the criterion of (1) narrowing of the MPD with enlargement of the pancreas, as determined by a review of an imaging study, is met together with the criterion of (2) an increase in serum markers (g-globulin, IgG, or IgG4) and/or the criterion of (3) pathology. In this study, the diagnosis of AIP was established by the presence of criteria 1 and 2 in 4 patients; criteria 1 and 3 in 3 patients; and criteria 1, 2, and 3 in 12 patients.

The f-AIP group included 19 patients (14 men and 5 women; age range, 41-75 years; mean age, 54 years) with 21 lesions who had undergone contrast-enhanced computed tomography (CE-CT) (n = 19), CE magnetic resonance imaging (MRI) (n = 11), and magnetic resonance cholangiopancreatography (MRCP) (n = 16). One patient had 3 lesions which were located at the pancreatic head, body and tail.

Identification of patients with focal pancreatic carcinoma was performed by reviewing patient records between March 2012 and December 2012 obtained from the hospital's pathology database. All 30 patients (21 men, 9 women; age range, 43-79 years; mean age, 58.2 years) with pathologically (histopathological examination of the surgically resected or biopsied tumor specimen) confirmed pancreatic ductal carcinoma (n = 30) were included in this study. All 30 patients had undergone CE-CT, 26 had undergone MRCP, and 12 had undergone CE MRI using extracellular MR contrast agents (Gd-DTPA).

Thirty patients (20 men, 10 women; age range, 41–68 years; mean age, 53 years) with normal pancreas were also recruited. They were confirmed by reviewing the radiological images and followed-up for more than 6 months. All 30 patients had undergone CE-CT.

CE-CT

In this study, all 79 patients underwent the triple-phase pancreatic CT protocol (Brilliance 16; PHILIPS Medical System) which included an unenhanced scan followed by triple-phase contrast-enhanced scans of the abdomen. The tube voltage was 120 kV, the tube current was 250 mA, and the rotation period was 0.75 s, A total of 90 mL of Ⅳ contrast material (iohexol, Omnipaque 300, GE Healthcare) containing 300 mg/mL iodine was intravenously administered as a bolus *via* a power injector. The injection rate was 3.5 mL/s. Images were obtained during the arterial, pancreatic and hepatic phases at 20, 40 and 80 s, respectively, after contrast medium injection. The median slice thickness for contrast-enhanced images was 3 mm.

MRI and MRCP

MR imaging and MR changiopancreatography were performed using a 1.5-T MR imaging system (AVANTO; SIEMENS Medical Systems) and a pre-contrast coil. Pre-contrast T1-weighted MR imaging [repetition time msec/echo time msec, 150 (R) 200/2.1, 4.2] with and without fat saturation and respiratory-triggered T2-weighted MR imaging (5000-8000/80-135) were performed, followed by dynamic fat-suppressed T1-weighted MR imaging (150-200/2.1) after administration of a gadolinium-based contrast agent. MRCP was performed with a single-shot fast spin-echo thick-slab technique (25000-30000/800-1000).

Imaging analysis

Three board-certified abdominal radiologists (with 9, 10 and 15 years of experience) reviewed all CT and MRI images retrospectively using a picture archiving and communication system (PACS) work station (General Electric Medical System). During analysis of the CT and MRI findings, all cases were randomly intermixed. The radiologists were blinded to the patients' clinical data, official reports, radiological examinations on other dates, and histopathological findings. Decisions were made by consensus.

For each patient, the radiologists were asked to make judgments on the following signs: (1) focal pancreatic enlargement and the location of the lesion; (2) capsule-like rim of the lesion; (3) localized irregular narrowing of MPD; (4) stricture of the distal common bile duct; and (5) other associated findings such as calcification, peripancreatic lymphadenopathy and vascular invasion.

When the lesion was confirmed, CT attenuation values of the f-AIP, PC, the apparently unaffected pancreatic parenchyma, and the normal pancreas were measured by one radiologist using a workstation (Advantage Version 4.2, GE Healthcare). The CT attenuation values were measured using unenhanced images and images obtained from arterial, pancreatic and hepatic phases after contrast administration. Following the placement of a region of interest (ROI) in each segment of the pancreas (head, body and tail), CT attenuation values of the pancreatic parenchyma were measured. The mean value of the three segments was used as the CT attenuation value of the pancreatic parenchyma. In f-AIP and PC patients, ROIs were placed both above the lesion and in the unaffected segments of the pancreas. The largest possible spherical ROI was marked, ruling out the pancreatic duct and partial volume averaging from the extrapancreatic structures. The smallest ROI was approximately 3 mm in diameter when the pancreas was atrophic. Delayed enhancement of the AIP and PC lesions was defined as the change in CT attenuation of the lesion between the arterial phase and the hepatic phase. CT attenuation values of the liver and spleen were similarly measured on unenhanced images (when available) and on images obtained in the arterial, pancreatic and hepatic phases.

The MRCP images were reviewed to identify changes in the common bile duct (CBD) and MPD. The MPD and CBD observations were classified into 1 of 3 categories: as displaying (1) normal appearance; (2) stenosis; and (3) complete obstruction (nonvisualization of the obstructed segment). The MPD upstream diameter was evaluated by a review of MRCP images. The MPD upstream diameter and presence of distal pancreatic atrophy were not evaluated in patients with lesions in the pancreatic tail, while stenosis of CBD was not evaluated for patients with lesions in the pancreatic head.

Statistical analysis

Statistical analysis was performed using the Fisher's exact test to compare the frequencies of imaging findings. The inter-reader agreement was evaluated by measurement of the kappa value. The mean CT attenuation value of the lesion in patients with f-AIP was compared with that of PC. Similarly, The mean CT attenuation values of unaffected segmental pancreas in f-AIP were compared with those of PC and normal pancreas. A comparison of the mean CT attenuation values of other organs (liver and spleen) was also performed in the three groups of patients.

Statistical analyses were performed with nonparametric tests due to the nongaussian distribution of the data and smaller sample sizes involved in some comparisons of interest. Wilcoxon's rank sum test was used to compare the CT attenuation values. When comparing two groups, the KruskalWallis test was performed before Wilcoxon's rank sum test. Fisher's exact test was applied to compare frequencies of delayed enhancement of the masses and focal enlarged segments. The diagnostic performance of attenuation value increase between the arterial and hepatic phase in the differentiation between f-AIP and PC was evaluated using receiver operating characteristic (ROC) analysis. From the ROC curves, the appropriate cutoff values were determined by selecting the point at which the Yoden index (sum of sensitivity + specificity - 1) was largest. All tests were two sided, and $P < 0.05$ was considered statistically significant. Statistical analysis was performed with SPSS software (version 18.0, SPSS).

RESULTS

The imaging characteristics of f-AIP and PC identified by reviewing the CE-CT and MRI/MRCP results are summarized in Tables 1 and 2.

Focal pancreatic enlargement

Of the 49 f-AIP and PC cases, the affected segments of pancreas differed in the extent of enlargement. Of the f-AIP cases, the affected sites of the pancreas were the head in 5 patients, the body in 5 patients, and the tail in 9 patients. Of the PC cases, these values were 16, 6 and 8, respectively. The sausage-shaped enlargement of the affected segments of the pancreas was observed in 11 patients with f-AIP, but not in PC cases. Atrophy of

Table 1 Comparison of imaging findings

Imaging features	Data of assessable patients		Kappa	*P*
	f-AIP (*n* = 19)	f-PC (*n* = 30)		
Sausage-shaped enlargement	0.58 (11/19)	0.03 (1/30)	0.58	< 0.001
Delayed homogeneous enhancement	1.00 (19/19)	0.1 (3/30)	0.88	< 0.001
Hypoattenuating capsule-like rim	0.63 (12/19)	0.03 (1/30)	0.64	< 0.001
Distal pancreatic atrophy	0.30 (3/10)	0.95 (20/22)	-0.55	< 0.001
MPD				
Normal	0 (0/19)	0 (0/30)		NS
Irregular narrowing	0.58 (11/19)	0.06 (2/30)	0.05	NS
Complete obstruction	0.42 (8/19)	0.93 (28/30)	-0.06	NS
CBD				
Normal	0.53 (10/19)	0.47 (14/30)	-0.36	0.027
Stenosis[1]	0.29 (4/14)	0 (0/14)	0.3	0.022
Complete obstruction	0 (3/19)	0.53 (16/30)	-0.38	< 0.01
MPD upstream dilation ≤ 5 mm	1.00 (10/10)	0.14 (5/22)	0.66	< 0.001
Affected location of pancreas				
Head	0.26 (5/19)	0.53 (16/30)	-0.08	NS
Body	0.26 (5/19)	0.2 (6/30)	-0.27	NS
Tail	0.47 (9/19)	0.27 (8/30)	0.11	NS
Other findings				
Vascular invasion	0 (0/19)	0.13 (4/30)	-0.17	NS
Pancreatic lymph node	0.16 (3/19)	0.87 (26/30)	-0.65	< 0.001
Calcification	0 (1/19)	0 (0/30)		NS

Data are percentages and numbers in parentheses refer to numbers of lesions. [1]The stenosis of common bile duct (CBD) was not evaluated for focal autoimmune pancreatitis (f-AIP) and pancreatic cancer (PC) patients with lesions in the pancreatic head. When the Kappa value was negative, the corresponding item was not suitable for differentiation. f-AIP: Focal autoimmune pancreatitis; MPD: Main pancreatic duct; NS: Not significant.

the pancreas was observed in 3 f-AIP and 20 PC cases whose lesions were located in the head and body.

Density or signal abnormalities

CE-CT scans showed hypoattenuated or isoattenuated lesions in the involved segments of the pancreas in 4 and 15 f-AIP cases on precontrast scans, respectively. In all f-AIP patients, the affected pancreas appeared uniformly enlarged with the absence of pancreatic clefts and with a sharp outline (Figures 1-3). AIP and PC lesions all showed decreased enhancement on arterial phase and delayed enhancement on pancreatic and hepatic phases, however, the degree of delayed enhancement in f-AIP was greater than that in PC (Figures 2 and 4, Table 2). MRI imaging showed that the lesion was isointense or slightly hypointense in T1WI and slightly hyperintense in T2WI (Figure 3A and B). The MR enhancement patterns were similar to those of CE-CT.

CBD and MPD Abnormalities

Of the AIP lesions (n = 21), irregular narrowing of the MPD was observed in 13 lesions (Figure 3E), and the remaining 8 lesions did not display this MPD abnormality. Bile duct dilatation was observed in 5 cases which included 4 patients who had lesions at the pancreatic head and 1 at the tail. The range of dilation was 9-14 mm (mean, 11 mm). The CBD showed a beak-like stricture (Figure 1C). In 3 patients with lesions in the pancreatic head, the upstream MPD was slightly dilated (less than 5 mm), distal pancreatic atrophy was not observed in 14 patients with lesions in the pancreatic body and tail.

Of the PC lesions (n = 30), no irregular narrowing of the MPD was observed. Bile duct dilatation was observed in all 8 patients with lesions located in the pancreatic head, and the distal CBD showed an abrupt interruption sign.

Capsule-like rim

Capsule-like rims were observed in f-AIP cases (n = 11), which were shown continuously or discontinuously as hypodense peripancreatic strands on the precontrast CT and delayed enhanced on the delayed phase of dynamic CT (Figure 2). On MR imaging, the capsule-like rim appeared as a T1WI isointense and T2WI hypointense area surrounding the pancreas (Figure 3A and B).

Other associated findings

Other associated findings included calcification (f-AIP, n = 1; PC, n = 0), peripancreatic lymphadenopathy (f-AIP, n = 3; PC, n = 26) and vascular invasion (f-AIP, n = 0; PC, n = 4).

Comparison of CT attenuation values

The results of the mean CT attenuation values of the f-AIP lesions and uninvolved segments in f-AIP patients and normal pancreas are shown in Table 2. The mean CT attenuation values of f-AIP lesions in enhanced phases were significantly higher than those of PC (P < 0.05, P < 0.05, P < 0.05) (Figure 5). The mean CT attenuation values of the f-AIP lesions were significantly lower than those of uninvolved pancreas and normal pancreas in the arterial and pancreatic phase of CT (P < 0.001, P < 0.001), however, there were no significant differences in the hepatic phase or unenhanced scanning (P = 0.4, P = 0.1). The mean CT attenuation values of normal and unaffected pancreatic parenchyma in the three groups showed no significant differences in the arterial, pancreatic, and hepatic phases (P = 0.1, P = 0.8, P = 0.2). The mean CT attenuation values of the liver and spleen were not significantly different between the three groups. When the attenuation value increase was equal or more than 28 HU this was considered diagnostic for f-AIP, and a sensitivity of 87.5%, specificity of 100% and an area under the ROC curve of 0.974 (95%CI: 0.928-1.021) were achieved (Figure 6).

DISCUSSION

Although the diagnosis of AIP has improved due to a growing awareness of the condition and proposed diagnostic criteria[18], there is no practical strategy to differentiate PC from f-AIP. One must distinguish between the two disorders to prevent unnecessary surgery or delayed

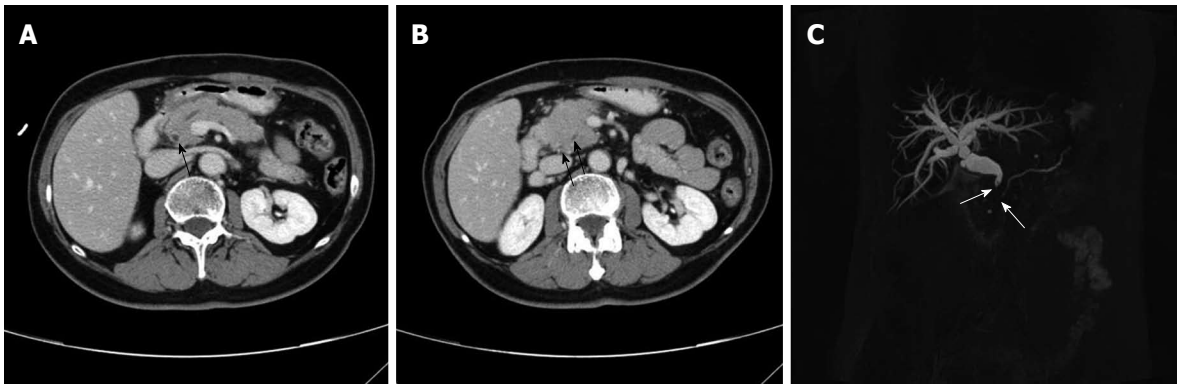

Figure 1 A 55-year-old woman with focal autoimmune pancreatitis of the pancreatic head. A, B: Contrast-enhanced computed tomography images obtained during enhanced phases showing enhanced thickening of the common bile duct (CBD) wall (A, black arrow), narrowing of the main pancreatic duct (MPD) and distal CBD (B, black arrows); C: Bile duct dilation and irregular narrowing of the MPD in the pancreatic head can be observed on the magnetic resonance cholangiopancreatography image (white arrows).

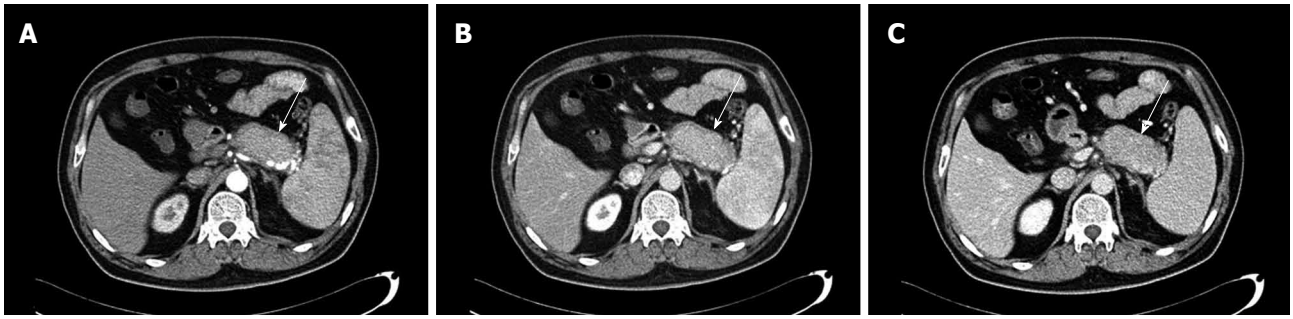

Figure 2 A 59-year-old man with focal autoimmune pancreatitis of the pancreatic tail. Contrast-enhanced computed tomography image obtained during the arterial phase (A, white arrow) showing that a hypoattenuating capsule-like rim can be observed around the pancreatic tail, which manifested delayed enhancement during the pancreatic phase (B, white arrow) and hepatic phase (C, white arrow). Attenuation values of the autoimmune pancreatitis lesion were 36, 51, 76 and 89 HU during the pre-contrast and enhanced phases, respectively.

Table 2 Mean computed tomography attenuation values

Group	*n*	Condition	Unenhanced scan (HU)	Arterial phase (HU)	Pancreatic phase (HU)	Hepatic phase (HU)
f-AIP	19	Lesions	36 ± 4	60 ± 7	85 ± 6	95 ± 7
		Uninvolved pancreas	42 ± 5	83 ± 10	105 ± 14	95 ± 7
		Liver	57 ± 4	68 ± 8	119 ± 15	100 ± 10
		Spleen	48 ± 3	111 ± 15	129 ± 15	96 ± 9
PC	30	Lesion	34 ± 5	48 ± 10	63 ± 15	63 ± 13
		Uninvolved pancreas	39 ± 6	76 ± 14	103 ± 15	87 ± 12
		Liver	58 ± 6	69 ± 9	108 ± 17	102 ± 10
		Spleen	46 ± 4	102 ± 19	141 ± 18	101 ± 7
Normal	30	Pancreas	44 ± 7	87 ± 12	105 ± 20	93 ± 10
		Liver	57 ± 7	65 ± 8	110 ± 18	99 ± 13
		Spleen	47 ± 4	107 ± 17	133 ± 23	99 ± 11

Data are computed tomography attenuation values (mean ± SD). f-AIP: Focal autoimmune pancreatitis; PC: pancreatic cancer.

initiation of corticosteroid therapy. A review of the CE-CT and MRI data indicated five imaging features of AIP: (1) delayed homogeneous enhancement; (2) hypoattenuateing capsule-like rim; (3) the absence of distal pancreatic atrophy; (4) irregular narrowing of the MPD; and (5) stenosis of the CBD in patients with lesions in the body or/and tail. The analysis also indicated that those imaging features could be used to differentiate AIP from PC with high accuracy.

Some studies have reported that the head of the pancreas is involved in most AIP cases[19,20], which is consistent with the study by Woo Ik Chang who showed that the affected site was the pancreatic head in 5 (62.5%) of 8 patients[13]. However, in our study, the affected site was the pancreatic head in only 4 patients (21.1%). This difference may be due to the following reasons: (1) the pancreatic head may not be the most commonly involved site in f-AIP; and (2) the number of patients studied may have been too small.

In our study, we identified the homogeneous good

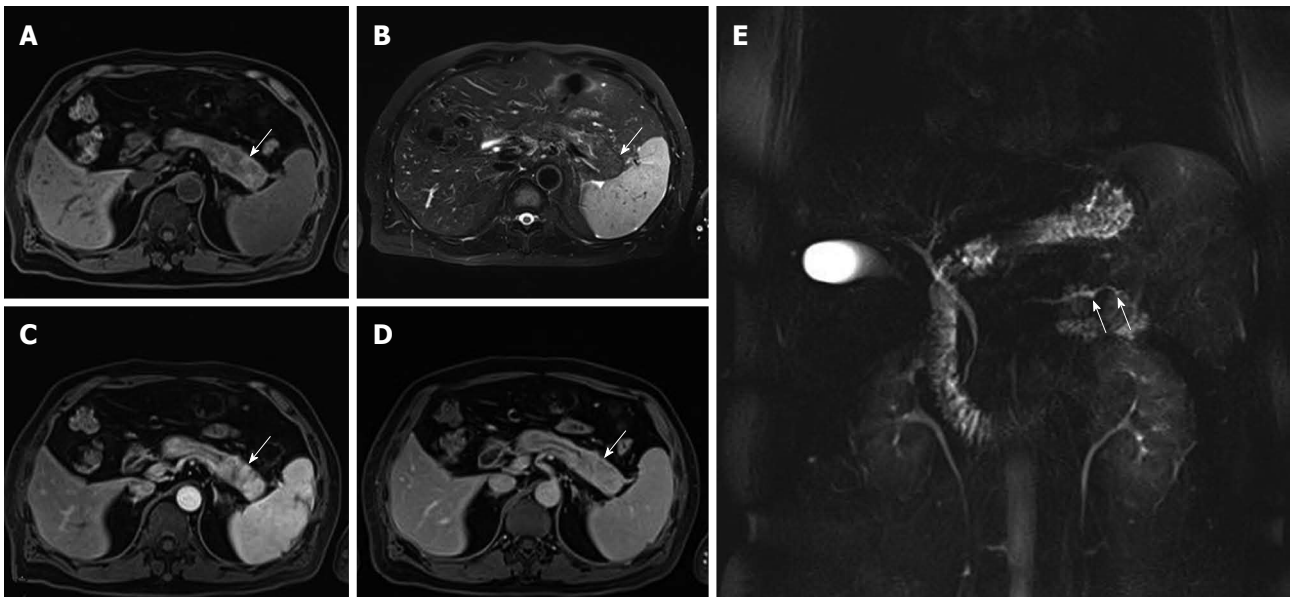

Figure 3 A 53-year-old man with focal autoimmune pancreatitis of the pancreatic tail. A-D: The lesion showed heterogenous T1WI hypointensity and heterogenous T2WI hyperintensity, which was delayed enhanced. The capsule-like rim appearing as a T1WI iso- or slight hyperintensity and a T2WI hypointensity area surrounding the pancreas (white arrow), which were delayed and moderately enhanced; E: Magnetic resonance cholangiopancreatography image shows the irregular narrowing main pancreatic duct in the pancreatic tail (white arrows).

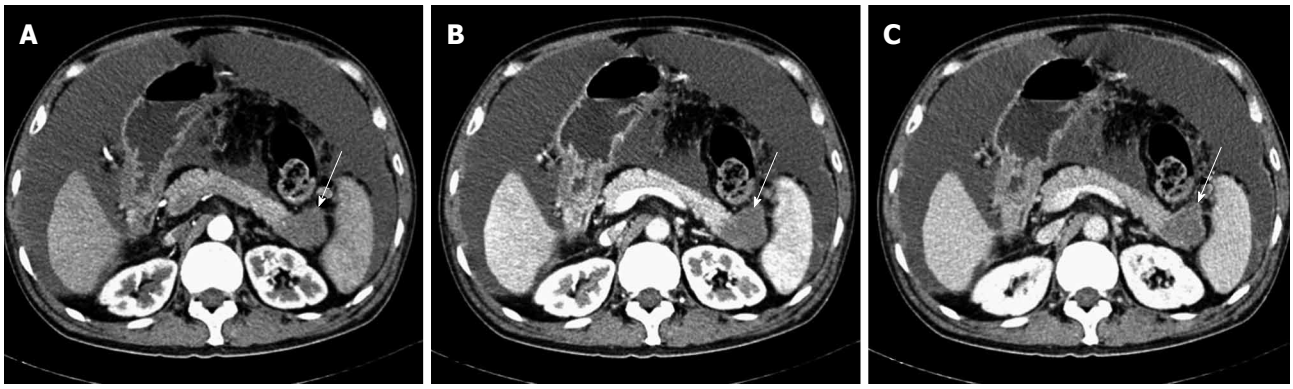

Figure 4 A 54-year-old man with pancreatic cancer of the pancreatic tail. Triple-phase computed tomography images show irregular enlargement of the pancreatic tail. Enhancement of the lesion is decreased (A, 39 HU) during arterial phase, and slightly increased enhancement during pancreatic phase (B, 48 HU) and hepatic phase (C, 62 HU), respectively (white arrows).

enhancement during the delayed phases as useful in differentiating between f-AIP and PC. Similarly, in the study by Kamisawa[12], delayed enhancement was observed in 17 of 17 (100%) AIP patients, while Wakabayashi *et al*[20] described delayed homogeneous enhancement in 9 of 9 (100%) AIP patients. In addition, Chang *et al*[13] described homogeneous enhancement during the hepatic phase in six of seven AIP patients. The consistency of delayed homogenous enhancement in this and previous studies shows one of the common imaging features of AIP, but the absence of a further quantitative analysis.

We found that the attenuation values of AIP were significantly higher than those of PC in the enhanced phases, lower than those of unaffected pancreatic parenchyma in the arterial and pancreatic phase, however, no significant differences were observed in the hepatic phase (95 ± 7 HU *vs* 95 ± 7 HU, 75 s). Takahashi found significantly higher CT attenuation values for AIP (90 ± 19 HU) than for pancreatic carcinoma (64 ± 19 HU) during the hepatic phase (60-70 s)[21]. They also found greater enhancement during the pancreatic phase (71 ± 22 HU *vs* 59 ± 20 HU), although the difference between the two groups was not statistically significant[10]. The difference between the CT attenuation values obtained in this study and those by Takahashi may be related to differences in the timing of scan acquisition, contrast injection rates and CT scanners.

Irregular narrowing of the MPD is one of the most important features of AIP. In our study, 79% of AIP patients (15 of 19) showed segmental irregular narrowing of the MPD, while the number was 6% (2 of 30) in PC patients. Moreover, no or minimal upstream dilatation can also be helpful to differentiate AIP from PC[10,22]. In the current study, upstream MPD dilatation (≥ 5 mm)

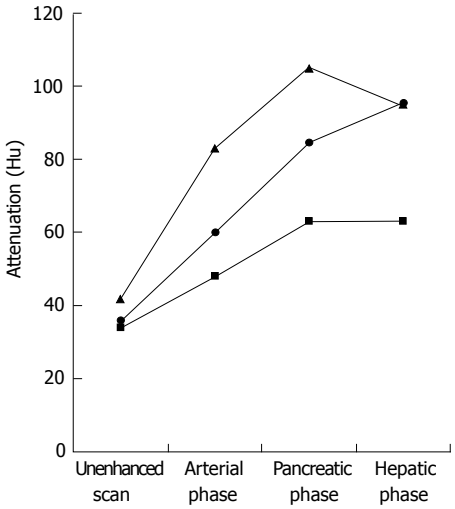

Figure 5 Graph shows mean computed tomography attenuation values of normal pancreatic parenchyma (triangle) and lesions in patients with focal autoimmune pancreatitis (circle) and pancreatic cancer (square) in relation to phase of contrast enhancement.

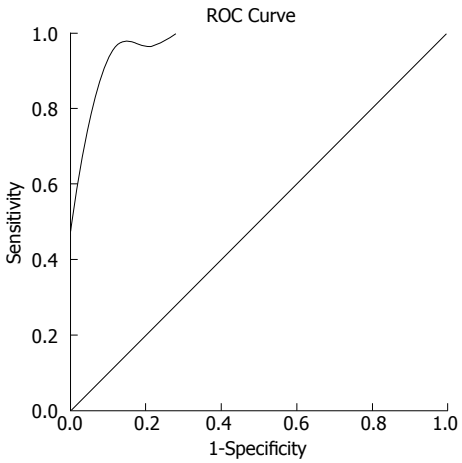

Figure 6 Receiver operating characteristic curve analysis result. Receiver operating characteristic (ROC) curve analysis revealed that when values were more than or equal 28 HU they were considered diagnostic for focal autoimmune pancreatitis, and a sensitivity of 87.5%, a specificity of 100%, and an area under the ROC curve of 0.974 (95%CI: 0.928-1.021) were achieved.

was observed in only 5% of patients (1 of 19), while the number was 86% in PC patients. Some studies reported that stricture of the distal CBD was often observed in AIP, and this occurred due to the combined effect of extrinsic compression by the inflamed pancreatic head and inflammatory changes in the CBD[10]. The f-AIP patients showed a smooth pattern, whereas the patients with PC showed an irregular pattern. In our study, CBD stricture of smooth pattern was seen in 8 of 19 patients, that is 4 of 5 patients with pancreatic head lesions and four with pancreatic tail lesions. Therefore, we suggest that the inflammatory changes in the CBD and AIP may be independent or have occurred one after another, and this might be useful for the diagnosis of f-AIP.

In the current study, the small number of patients may not represent all the characteristics of f-AIP according to radiological features only. Thus, we tried to identify radiological findings to diagnosis f-AIP, however, these findings were insufficient to exclude PC. Therefore, we recommend that IgG4 should be tested and histological findings should be obtained where possible to discriminate f-AIP from PC. We believe that future studies with more patients will clarify and validate the radiological features of f-AIP.

In conclusion, this study found that the analysis of a combination of imaging findings, either of delayed enhancement with more than 28 HU, or of three imaging findings (1) focal pancreatic enlargement with a capsule-like rim; (2) irregular narrowing of the MPD; and (3) stricture of the CBD in patients with lesions (not located in the pancreatic head) contribute to improve the diagnostic accuracy of f-AIP thus avoiding unnecessary surgery.

COMMENTS

Background

Autoimmune pancreatitis (AIP), a rare form of chronic pancreatitis (CP), can be classified as the diffuse form and the focal form. Although AIP is well-known among radiologists, focal AIP (f-AIP) is still very difficult to differentiate from pancreatic cancer (PC).

Research frontiers

F-AIP has a variety of manifestations and is very difficult to differentiate from PC. Improving the diagnostic accuracy can help avoid unnecessary surgery in f-AIP patients.

Related publications

The analysis of combined imaging with computed tomography and magnetic resonance to improve the diagnostic accuracy of differentiating f-AIP from PC has rarely been reported.

Innovations and breakthroughs

This is the first study to report that the triple-phase enhancement pattern of f-AIP is different from that of PC. Three imaging features are more frequently found in f-AIP, including focal pancreatic enlargement with a capsule-like rim, irregular narrowing of the main pancreatic duct and stricture of the common bile duct in patients with lesions not located in the pancreatic head. The combination of these findings could further improve the diagnostic accuracy of f-AIP and avoid unnecessary surgery.

Applications

The study results suggest that a combination of imaging findings will help improve the diagnostic accuracy of f-AIP and avoid unnecessary surgery.

Peer review

It is a good clinical study in which the authors analyzed the radiological characteristics of f-AIP. The results are interesting and suggest that the combination of triple-phase enhancement pattern and imaging features of f-AIP can help for the differential diagnosis of f-AIP from PC.

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BRIEF ARTICLE

Fast-track surgery could improve postoperative recovery in radical total gastrectomy patients

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Author contributions: Feng F, Ji G and Li JP contributed equally to this work; Feng F and Zhao QC designed the study and wrote the manuscript; Ji G, Li JP and Liu XN performed all the operations; Li XH and Shi H were mainly in charge of perioperative management of patients; Zhao ZW and Wu GS were mainly in charge of evaluating postoperative outcomes, discharge, follow-up and data analysis.

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Abstract

AIM: To assess the impact of fast-track surgery (FTS) on hospital stay, cost of hospitalization and complications after radical total gastrectomy.

METHODS: A randomized, controlled clinical trial was conducted from November 2011 to August 2012 in the Department of Digestive Surgery, Xijing Hospital of Digestive Diseases, the Fourth Military Medical University. A total of 122 gastric cancer patients who met the selection criteria were randomized into FTS and conventional care groups on the first day of hospitalization. All patients received elective standard D2 total gastrectomy. Clinical outcomes, including duration of flatus and defecation, white blood cell count, postoperative pain, duration of postoperative stay, cost of hospitalization and complications were recorded and evaluated. Two specially trained doctors who were blinded to the treatment were in charge of evaluating postoperative outcomes, discharge and follow-up.

RESULTS: A total of 119 patients finished the study, including 60 patients in the conventional care group and 59 patients in the FTS group. Two patients were excluded from the FTS group due to withdrawal of consent. One patient was excluded from the conventional care group because of a non-resectable tumor. Compared with the conventional group, FTS shortened the duration of flatus (79.03 ± 20.26 h *vs* 60.97 ± 24.40 h, $P = 0.000$) and duration of defecation (93.03 ± 27.95 h *vs* 68.00 ± 25.42 h, $P = 0.000$), accelerated the decrease in white blood cell count [$P < 0.05$ on postoperative day (POD) 3 and 4], alleviated pain in patients after surgery ($P < 0.05$ on POD 1, 2 and 3), reduced complications ($P < 0.05$), shortened the duration of postoperative stay (7.10 ± 2.13 d *vs* 5.68 ± 1.22 d, $P = 0.000$), reduced the cost of hospitalization (43783.25 ± 8102.36 RMB *vs* 39597.62 ± 7529.98 RMB, $P = 0.005$), and promoted recovery of patients.

CONCLUSION: FTS could be safely applied in radical total gastrectomy to accelerate clinical recovery of gastric cancer patients.

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Key words: Fast-track surgery; Gastric cancer; Radical total gastrectomy; Perioperative care; Outcomes

Core tip: Fast-track surgery (FTS) is a promising program for surgical patients, and has been applied in several surgical diseases. The value of FTS in radical distal gastrectomy has been demonstrated recently, but the safety and efficacy of FTS for radical total gastrectomy requires further evaluation. The present study showed that FTS was feasible for perioperative care in radical total gastrectomy. Compared with conventional care, FTS could shorten the duration of flatus and defeca-

tion, accelerate the decrease in white blood cell count, decrease postoperative complications, shorten the postoperative stay, reduce the cost of hospitalization, and promote postoperative recovery of patients.

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INTRODUCTION

Fast-track surgery (FTS) was initiated by the Danish surgeon H Kehlet in the field of elective colorectal surgery in the 1990s[1,2], and has rapidly gained popularity around the world because of its significant benefits and safety[3]. The core elements of FTS include: epidural or regional anesthesia, perioperative fluid management, minimally invasive techniques, optimal pain control, early initiation of oral feeding and early mobilization[4]. The combination of these approaches has led to a significant reduction in complication rates, morbidity and mortality rates, duration of hospital stay and costs of hospitalization, and finally, greatly improved postoperative recovery[5-7]. In recent years, FTS has been applied in several surgical diseases, include radical prostatectomy[8], cardiac surgery[9], total knee replacement[10], cesarean section[11], coronary artery bypass grafting[12], it has also been used for specific procedures in children[13] and the elderly[14].

Gastric cancer is the fourth most common cancer worldwide but the second leading cause of cancer mortality[15], and it is more common in men and in developing countries. Up to now, surgery has been the most common treatment. For radical gastrectomy, conventional elective gastric resection and perioperative care are associated with a morbidity of 20%-46%, a mortality of 0.8%-10%[16] and a postoperative hospital stay of 8-13 d[17]. The high rate of complications leads to prolonged duration of hospital stay and increased costs of hospitalization.

The value of FTS in radical distal gastrectomy has been demonstrated recently[18,19], but the safety and efficacy of FTS in radical total gastrectomy still requires further evaluation. Therefore, we performed a slightly modified fast-track protocol in gastric cancer patients in our department. We evaluated the feasibility and safety of FTS in gastric cancer patients through a prospective, randomized comparative study.

MATERIALS AND METHODS

Patients

This study was performed in Xijing Hospital of Digestive Diseases affiliated to the Fourth Military Medical University from November 2011 to August 2012. Selection criteria were: (1) diagnosis of gastric cancer based on clinical symptoms, imaging and pathology; (2) age between 18 and 75 years; (3) no preoperative radiotherapy or chemotherapy; (4) no distant metastasis; (5) no history of primary diabetes mellitus, bowel obstruction, severe cardiopulmonary diseases, and immune related diseases; (6) no pregnancy or breast feeding; (7) an American Society of Anesthesiologists (ASA) score of Ⅰ or Ⅱ; (8) undergoing elective standard D2 total gastrectomy; and (9) written informed consent was obtained from the patient and the family. Gastric cancer patients meeting the selection criteria were randomly divided into a FTS group and a conventional care group immediately after admission. The sample size of 122 patients (61 cases in each group) was calculated with an alpha level of 0.05 and 90% power for primary endpoints.

This study was approved by the Ethics Committee of Xijing Hospital. This study was registered under chictr.org, identifier number ChiCTR-TRC-11001440.

Randomization and implementation

All the patients were clearly informed about the aims and details of the present study and signed consent forms. Random numbers were generated by computer. Eligible patients were randomly assigned in a 1:1 ratio. The specially trained investigator prepared allocation envelopes for the doctors of the enrolled patients. The investigator did not contact the patients throughout the clinical trial. The doctors and nurses administering the interventions and collecting the data had no role in the randomization process. Two specially trained doctors who were blinded to the treatment were in charge of evaluating postoperative outcomes, discharge and follow-up.

Interventions

The patients were admitted to the hospital 1-2 d before surgery. A slightly modified fast-track protocol proposed by Kehlet *et al*[20] was used in the present FTS group. Patients in the conventional surgery group received conventional perioperative care. Details of the interventions are listed in Table 1. Both groups were protocol-driven, with appropriate protocol details for patients, surgeons and nurses to ensure compliance.

Discharge criteria and readmission

Patients were considered dischargeable postoperatively if they met the following criteria: normal body temperature, pain controlled with oral analgesics, normal mobilization, no discomfort, normal oral diet, no parenteral nutrition, normal gastrointestinal function (normal flatus and defecation), Karnofsky Performance Status Scale score exceeding 80, and willing to go home.

After discharge, the patients were followed up by our specially trained surgeons through telephone within the first 24 h and once per week for 4 wk, and the patients could also contact us if they had any discomfort. The patients were readmitted if any of the following occurred:

Table 1 Comparison of fast-track surgery and conventional perioperative intervention protocols

Perioperative intervention	Conventional	Fast-track surgery
Diet before surgery	No intake of food and drink after supper the day before surgery	Intake of 1000 mL 14% carbohydrate drink 12 h before and 350 mL 14% carbohydrate drink 3 h before surgery.
Anesthesia	Tracheal intubation and general anesthesia	Tracheal intubation and general anesthesia
Thermal insulation during operation	No thermal insulation, room temperature was maintained at 22 ℃	Thermal insulation of the body and extremities, body temperature was maintained at 36 ℃
Operation procedure	Standard laparotomy approach	Standard laparotomy approach
Placement of abdominal drainage	Use of abdominal drainage tube	No routine use of abdominal drainage tube
Analgesia after operation	Standard use of patient-controlled analgesic pump	Infiltration of surgical wounds with ropivacaine at the end of surgery and 24 h after surgery. Oral intake of 200 mg celecoxib twice daily
Mobilization after operation	Mobilize out of bed on patients' own request	Encourage patients to mobilize out of bed
Diet after operation	Oral intake initiated after flatus (following a stepwise plan from water to other liquids to semi-fluids to normal food)	Oral intake of 500-1000 mL glucose saline on the day of surgery. Intake of 2000-3000 mL liquid food containing 1000 kcal to 1200 kcal per day from the 1st day after surgery
Intravenous nutrition after operation	Infusion of glucose saline and amino acid injection *iv* on the day of surgery. Infusion of parenteral nutrition (25 kcal/kg of body weight) *iv* before oral intake. Appropriate level of *iv* fluid intake based on the volume of liquid intake and output, and physiological need	Infusion of parenteral nutrition *iv* if oral intake is not adequate. Appropriate level of *iv* fluid intake based on the volume of liquid intake and output, and physiological need
Removal of nasogastric tube	Removal of nasogastric tube after flatus	Removal of nasogastric tube within 24 h after surgery
Removal of urine catheter	Removal of urine catheter on the 3rd or 4th day after surgery	Removal of urine catheter within 24 h after surgery
Antibiotics	Standard use of antibiotics for 3 d after surgery	Standard use of antibiotics before and once after surgery

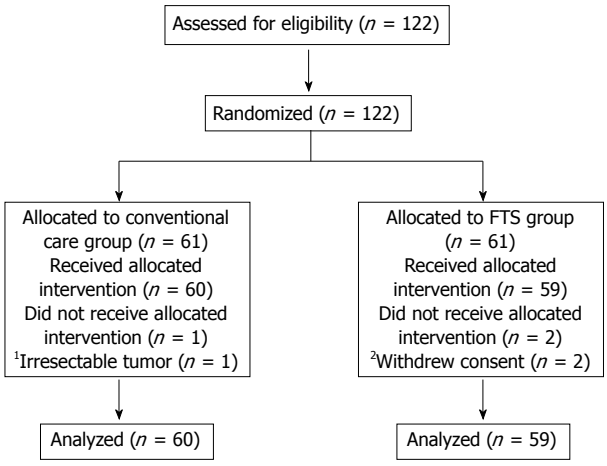

Figure 1 Flow diagram of the randomized control trial designed to compare the safety and efficacy of fast-track surgery and conventional care groups. [1]One patient had an irresectable tumor in theconventional care group; [2]Two patients withdrew consent in the fast-track surgery (FTS) group. All three patients were excluded from the analysis.

hyperpyrexia, abdominal pain, bowel obstruction, gastrointestinal hemorrhage, malnutrition, infection and poor healing of the wound.

Data collection

The primary clinical endpoints were the duration of hospital stay and the cost of hospitalization. The second clinical endpoints were incidence of complications such as pneumonia, surgical site infection, abdominal infection, anastomotic leak, and bowel obstruction. We recorded preoperative data on age, sex, body mass index (BMI), nutritional risk screening (NRS) 2002 score, ASA score, differentiation status, TNM classification, white blood cell (WBC) count, hemoglobin, albumin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Surgical-related data such as operation time and blood loss were also recorded. Postoperative data such as timing of first flatus and defecation, duration of hospital stay, the cost of hospitalization and complications were recorded. WBC was measured from postoperative day (POD) 1 to POD 5. Pain intensity was evaluated from POD 1 to POD 5 using a visual analog scale (VAS).

Statistical analysis

Data were processed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, United States). Numerical variables were expressed as the mean ± SD unless otherwise stated. Differences between the two groups were tested using a two-tailed Student *t* test. Discrete variables were analyzed using the χ^2 test or Fisher's exact test. A P value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics

A total of 119 patients finished the study, including 60 patients in the conventional care group and 59 patients in the FTS group. Two patients were excluded from the FTS group after withdrawing consent. One patient was excluded from the conventional care group because of an irresectable tumor (Figure 1). The preoperative baseline characteristics of the two groups are compared in Table 2. There were no significant differences between the two groups in age, sex, BMI, NRS 2002 score, ASA score, differentiation status, TNM classification, WBC count, he-

Table 2 Comparison of baseline characteristics of the two groups (mean ± SD)

Characteristics	Conventional	Fast-track surgery	*P* value
Age, yr	55.79 ± 10.06	54.98 ± 11.35	0.682
Sex			0.689
Male/female	44/16	41/18	
BMI	21.01 ± 1.78	22.44 ± 3.51	0.061
NRS 2002 score	0.81 ± 1.10	1.08 ± 1.41	0.424
ASA score			0.364
Ⅰ/Ⅱ	1/59	3/56	
Differentiation status			0.857
Well differentiated	6	4	
Moderately differentiated	20	17	
Poorly differentiated	34	38	
TNM classification			0.324
Ⅰ/Ⅱ/Ⅲ	8/31/2021	14/12/33	
White blood cell	6.20 ± 1.74	6.05 ± 2.08	0.671
Hemoglobin, g/L	133.36 ± 22.03	130.65 ± 22.41	0.52
Albumin, g/L	44.42 ± 4.89	42.83 ± 4.65	0.082
ALT	17.91 ± 11.35	21.29 ± 15.55	0.195
AST	21.84 ± 11.46	25.83 ± 17.00	0.151
Operation time, min	242.38 ± 72.89	226.11 ± 65.87	0.214
Blood loss, mL	221.17 ± 122.55	230.55 ± 171.82	0.735

BMI: Body mass index; ASA: American Society of Anesthesiologists; NRS: Nutritional risk screening; TNM: Tumor node metastases; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 3 Comparison postoperative pain intensity and white blood cell count between the two groups (mean ± SD)

Time	Conventional	Fast-track surgery	*P* value
Postoperative pain intensity			
POD 1	5.41 ± 1.45	4.32 ± 1.65	0.000
POD 2	4.43 ± 1.54	3.39 ± 1.65	0.001
POD 3	3.63 ± 1.48	2.76 ± 1.36	0.002
POD 4	3.02 ± 1.45	2.51 ± 1.87	0.119
POD 5	2.21 ± 1.39	2.30 ± 1.56	0.789
White blood cell count			
POD 1	14.81 ± 5.34	14.55 ± 5.04	0.793
POD 2	15.36 ± 5.36	12.26 ± 4.78	0.002
POD 3	11.80 ± 4.80	9.35 ± 3.83	0.005
POD 4	8.56 ± 3.70	7.52 ± 3.57	0.223
POD 5	6.37 ± 2.34	6.91 ± 3.34	0.684

POD: Postoperative day.

moglobin, albumin, ALT, AST, operation time and blood loss (all $P > 0.05$).

Pain intensity

Pain intensity was evaluated from POD 1 to POD 5 in the two groups (Table 3). VAS analysis showed that pain intensity of patients in the FTS group was significantly lower than that of patients in the conventional care group on POD 1-3 ($P < 0.05$).

White blood cell count

The WBC counts of patients in the two groups were measured in the morning of POD 1 to POD 5 (Table 3). The WBC count in the conventional care group and FTS group were both elevated on POD 1. Although the WBC count in the conventional care group continued to rise on POD 2, the WBC count in the FTS group began to drop ($P < 0.05$). The WBC count in the conventional care group began to drop on POD 3, but was significantly higher than in the FTS group ($P < 0.05$).

Table 4 Comparison clinical outcomes and postoperative complications between the two groups

	Conventional	Fast-track surgery	*P* value
Clinical outcomes			
First flatus, h	79.03 ± 20.26	60.97 ± 24.40	0.000
First defecation, h	93.03 ± 27.95	68.00 ± 25.42	0.000
Postoperative stay, d	7.10 ± 2.13	5.68 ± 1.22	0.000
Cost of hospitalization, RMB	43783.25 ± 8102.36	39597.62 ± 7529.98	0.005
Postoperative complications			
Total cases	17	6	0.019
Pneumonia	10	5	0.269
Incision infection	3	1	0.619
Urinary infection	1	0	1.000
Abdominal infection	1	0	1.000
Gastric retention	0	0	
Anastomotic leak	0	0	
Deep-vein thrombosis	0	0	
Ileus	1	0	1.000
Reoperation	1	0	1.000
Readmission	0	0	
Mortality	0	0	

Outcomes

The outcomes were summarized in Table 4. Compared with the conventional care group, the patients in the FTS group showed significantly accelerated recovery of gastrointestinal function in terms of time to first flatus and first defecation ($P < 0.05$). The duration of postoperative stay of the FTS group was significantly shorter than that of the conventional care group ($P < 0.05$) and the cost of hospitalization was also significantly lower ($P < 0.05$).

Complications and readmissions

Table 4 summarizes the complications and readmissions in each group. The overall complication rate in the FTS group (10.17%) was significantly lower than in the conventional group (28.33%, $P = 0.019$). In the conventional care group, 10 patients suffered from pneumonia, 3 patients suffered from incision infection, 1 patient experienced urinary infection, 1 patient experienced abdominal infection, and 1 patient underwent reoperation because of ileus. In the FTS group, 5 patients suffered from pneumonia and 1 experienced incision infection. All the patients were cured by surgery or conservative treatment.

DISCUSSION

The aim of the present study was to evaluate the safety, efficacy and outcome of FTS protocol employed in the perioperative treatment of gastric cancer in comparison with conventional perioperative treatment. The data of the present study showed that the FTS protocol was feasible for perioperative care of gastric cancer patients who underwent radical total gastrectomy. Compared with

conventional care, FTS could shorten the duration of flatus and defecation, accelerate the decrease in WBC, decrease postoperative complications, shorten the duration of postoperative stay, reduce the cost of hospitalization, and eventually promote postoperative recovery of the patients.

Optimal pain control is very important. Pain can not only result in stress[21], but also affects the mobilization of patients after surgery. Early mobility or activity is recognized as a critical step in fast-track care. Bed rest not only increases muscle loss and insulin resistance, but also decreases pulmonary function and supply of oxygen to tissues[22]. It has been reported that opioids may result in nausea, vomiting and fatigue that counteract the benefits of FTS[23]. Therefore, routine use of opioids was avoided in the FTS group. In our present study, the infiltration of surgical wounds with ropivacaine and oral intake of celecoxib were applied instead of a patient-controlled analgesia pump. Pain intensity was evaluated from POD 1 to POD 5 after surgery using the VAS. The results showed that VAS in the FTS group was significantly lower than that of conventional care group. This indicated that ropivacaine combined with celecoxib had a better analgesic effect than an analgesic pump, and the better analgesic effect in the FTS group ensured a longer duration of mobilization out of bed.

Conventionally, the duration of antibiotic use is 2-3 d after gastrectomy. In the present study, the antibiotics were only applied before and after surgery in the FTS group (Table 1). We noticed that even with shorter use of antibiotics in the FTS group, the WBC decreased earlier and faster than in the conventional postoperative care group.

Nasogastric tubes have been used traditionally for decompression after gastric surgery and remain a routine part of postoperative care in many centers. Nasogastric tubes are often left for several days until the first flatus after gastric resection. This is based on the rationale that this can prevent aspiration, and reduce the risk of intestinal obstruction and anastomotic leak in clinical practice. Previous studies have shown that the small intestine might return to normal enterocinesia 6 h after abdominal surgery[24]. Recent studies comparing nasogastric decompression *vs* no decompression demonstrated that a gastric tube may induce pulmonary complications after gastric cancer surgery[25,26] and prolong the time to first flatus with no difference in anastomotic leak rate[27]. Therefore, placement of a nasogastric tube is unnecessary. In our present study, a nasogastric tube was not routinely used in FTS group and was removed within th 24 h after surgery.

Multiple studies have demonstrated that drains are unnecessary after gastrointestinal surgery[28]. The placement of abdominal drainage is prone to increased feelings of pain, intra-abdominal fluid collection, infection, internal organ injuries and risk of fistulas, resulting in delayed recovery[17]. Alvarez Uslar *et al*[29] reported that operative morbidity and hospital stay were significantly higher in patients who underwent total gastrectomy with abdominal drains than that in patients without drains. However, we refrain from abolishing use of abdominal drains for total gastrectomy in China. Since all the patients received D2 total gastrectomy, the degree of lymph node dissection could lead to a higher risk of chyle leakage. Therefore, the use of drains after total gastrectomy continues to be an issue for debate in the development of FTS.

An early postoperative oral diet can hasten the return of gut function, protect gut mucosal barrier function, and enhance portal circulation[30]. Early enteral nutrition with dietary fiber can alleviate intestinal barrier dysfunction and decrease the incidence of bacterial translocation[31]. Although early enteral nutrition increases the incidence of vomiting and flatulence, a series of reports showed that it can reduce the risk of postoperative complications and mortality[32], facilitate postoperative restoration without increasing the incidence of fistulas[33], and be safety applied in gastrectomy[34]. In the present study, the majority patients in the FTS group well tolerated an early oral diet or enteral nutrition by jejunal feeding tube. We noticed that nausea and vomiting was rare, but abdominal distension did occur in some patients, the symptoms only lasted for a short time based on adequate mobilization out of bed and did not result in severe complications.

It is reported that the postoperative hospital stay of gastric patients could be deceased to 3.8 d in FTS group[35]. In the present study, the mean postoperative stay of patients in FTS group was 5 d, which was longer than that reported in the literature. We found that the traditional Chinese concepts of patients are the main obstacles. They believe that surgery could cause great damage to their bodies, and they could not recover in a short time. Thus, they worried about their safety after discharge from hospital. Therefore, preoperative patient instruction and education is crucial to the outcome of FTS[36]. It will let the patients fully understand the safety, efficacy and benefits of FTS, and guarantee the compliance of patients with medical and FTS protocols.

From the view of the doctors, concern about anastomotic leakage was the main reason which affected early discharge. A series of studies showed that the FTS protocol did not increase the incidence of anastomotic leakage[37], and revealed that education of FTS concepts was also very critical for doctors. Compliance with the FTS protocol is the main factor influencing the outcome of FTS[38]. Thus, we established a study group made up of a researcher, surgeons, anesthesiologists and nurses. We periodically conducted meetings with all staff about the details of FTS, in order to ensure the quality of the study.

The limitation of our present study was the inadequate adherence to the FTS protocol. Epidural analgesia was critical for FTS. Intraoperative application and postoperative use of epidural analgesia could block sympathetic activation to outside stimulation, inhibit hormone secretions of the hypothalamic-pituitary-adrenal axis, and finally attenuate responses to stress[39]. In our present study, tracheal intubation and general anesthesia were applied in both groups, which may partially decrease the

efficacy of FTS.

The present study indicates that FTS could promote postoperative recovery, decrease the rate of complications, shorten the duration of hospital stay, and reduce the cost of hospitalization. Our data indicate that FTS is a safe and efficient perioperative management strategy in patients undergoing radical total gastrectomy. Along with the further understanding of stress and development of FTS perioperative care, FTS could probably be safely applied in critically ill patients and emergency surgery, and major operations such as tumor resection may become day procedures in the near future.

COMMENTS

Background

Fast-track surgery (FTS) is a promising comprehensive program for surgical patients in elective surgery. In recent years, FTS has been applied in several surgical diseases, include radical prostatectomy, cardiac surgery, total knee replacement, cesarean section, and coronary artery bypass grafting. It has also been used for specific procedures in children and elderly. The value of FTS in radical distal gastrectomy has been demonstrated recently, but the safety and efficacy of FTS in radical total gastrectomy still requires further evaluation.

Research frontiers

The value of FTS in radical distal gastrectomy has been demonstrated recently. Chen *et al* evaluate the safety and effectiveness of fast-track surgery combined with laparoscopy-assisted radical distal gastrectomy for gastric cancer. They found that a combination of FTS and laparoscopy-assisted radical distal gastrectomy in gastric cancer is safe, feasible, and efficient and can improve nutritional status, lessen postoperative stress, and accelerate postoperative rehabilitation.

Innovations and breakthroughs

The present study showed that the FTS protocol was feasible for perioperative care of gastric cancer patients. Compared with conventional care, FTS could shorten the duration of flatus and defecation, accelerate the decrease in white blood cell count, decrease postoperative complications, shorten the duration of postoperative stay, reduce the cost of hospitalization, and eventually promote postoperative recovery of patients.

Applications

The data indicate that FTS is a safe and efficient perioperative management strategy in patients undergoing radical total gastrectomy. Along with further understanding of stress, and development of FTS perioperative care, FTS could probably be safely applied in critically ill patients and emergency surgery, and major operations such as tumor resection may become day procedures in the near future.

Terminology

FTS: Fast-track surgery, initiated by the Danish surgeon H Kehlet in the field of elective colorectal surgery in the 1990s, is a promising comprehensive program for surgical patients in elective surgery; the visual analogue scale is a psychometric response scale which can be used in questionnaires. It is a measurement instrument for subjective characteristics that cannot be directly measured.

Peer review

This was a good study in which the authors indicates that FTS could promote postoperative recovery, decrease rate of complications, shorten duration of hospital stay, and reduce the cost of hospitalization. However, the author should think about the reason of more pneumonia in conventional care group although it is not significant.

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BRIEF ARTICLE

Hepatocellular carcinoma: Clinical study of long-term survival and choice of treatment modalities

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Abstract

AIM: To analyze the prognostic factors of 5-year survival and 10-year survival in hepatocellular carcinoma (HCC) patients, and to explore the reasons for long-term survival and provide choice of treatment modalities for HCC patients.

METHODS: From January 1990 to October 2012, 8450 HCC patients were included in a prospective database compiled by the Information Center after hospital admission. Long-term surviving patients were included in a 10-year survival group (520 patients) and a 5-year survival group (1516 patients) for analysis. The long-term survival of HCC patients was defined as the survival of 5 years or longer. Clinical and biologic variables were assessed using univariate and multivariate analyses. The survival of patients was evaluated by follow-up data.

RESULTS: The long-term survival of HCC patients was associated with the number of lesions, liver cirrhosis and Child-Pugh classification. It was not found to be associated with tumor diameter, histological stage, and pretreatment level of serum α-fetoprotein. The differences in clinical factors between the 5-year survival and the 10-year survival were found to be the number of lesions, liver cirrhosis, Child-Pugh classification, and time elapsed until first recurrence or metastasis. The survival period of different treatment modalities in the patients who survived for 5 years and 10 years showed significant differences: (in order of significance) surgery alone > surgery-transcatheter arterial chemoembolization (TACE) > TACE-radiofrequency ablation (RFA) > TACE alone > surgery-TACE-RFA. The 10-year survival of HCC patients was not associated with the choice of treatment modality.

CONCLUSION: This retrospective study elucidated survival outcomes, prognostic factors affecting survival and treatment modalities in HCC patients.

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Key words: Hepatocellular carcinoma; Surgery; Radiofrequency ablation; Transcatheter arterial chemoembolization; Statistical analysis; Clinical study

Core tip: This manuscript was a retrospective analysis and it revealed that the long-term survival of hepatocellular carcinoma (HCC) patients was associated with the number of lesions, liver cirrhosis and Child-Pugh classification, whiletumor diameter, histological stage, and pretreatment level of serum α-fetoprotein

were not related. Conditions for long-term survival of HCC patients were: age over 50 years, no cirrhosis, a uninodular lesion, no vessel invasion, tumor-node-metastasis stage Ⅰ or Ⅱ, Child-Pugh classification Class A, and appropriate treatment. The best treatment modality for more than 10 years survival compared with 5 years survival were surgery alone > surgery-transcatheter arterial chemoembolization (TACE) > TACE-radiofrequency ablation (RFA) > TACE alone > surgery-TACE-RFA.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. The prognostic factors of primary HCC have been studied by many researchers worldwide. Several reports conducted in the 1990s in both Eastern and Western centers have documented a 5-year overall survival rate of 26% to 44% after HCC resection[1-5]. Studies performed from 1975 to the present[6-15] showed that age, gender, metastasis, bilirubin levels, ascites, tumor thrombus of the portal vein, neoplasm staging, Child-Pugh classification, and serum levels of γ-glutamyltransferase (GGT), alkaline phosphatase, and lactate dehydrogenase may all have significant effects on the prognosis of HCC patients.

There are many different modes of HCC treatment and types of primary HCC. The effects of treatment and survival rate have seen only limited improvements[16-21]. Curative surgical therapies have shown the best long-term survival rates. However, most patients do not meet the selection criteria for these treatments. Surgical resection is not available to HCC patients with severe liver cirrhosis, multiple lesions located in different parts of the liver, or lesions located near a large vein or the junctions of large portal veins[22,23]. For this reason, effective, minimally invasive therapeutic options are essential to improving the prognosis in HCC patients. Existing minimally invasive local treatments for primary HCC include radiofrequency ablation (RFA), microwave ablation, cryoablation, transcatheter arterial chemoembolization (TACE), percutaneous ethanol injection therapy, and high-intensity focused ultrasound ablation. In recent years, preliminary clinical trials have suggested that targeted drugs may have certain curative effects in the treatment of HCC[24-26].

This study discusses the factors relevant to duration of survival in these primary HCC patients. The method of treatment, the tumor's general prognostic factors, and the tumor's own characteristics were found to determine duration of survival. Therefore, the purpose of the present study was to analyze and compare the prognostic factors between 5-year survival and 10-year survival groups, to explore the clinical reasons for long-term survival and the choice of treatment modalities for HCC patients.

MATERIALS AND METHODS

Patients

From January 1990 to October 2012, 8450 HCC patients from Sun Yat-Sen University Cancer Center, First Affiliated Hospital of Jinan University, and Guangdong General Hospital were included in a prospective database after hospital admission. Long-term surviving patients were those who survived more than 5 years and they were identified and included in a 10-year survival group (520 patients) and 5-year survival group (1516 patients)for analysis. The long-term survival of HCC patients was defined as survival of 5 years or longer. This study was approved by the internal review board and ethics committee of the hospital. Informed consent was not required because of the retrospective and anonymous nature of the study, although these data were derived from prospective statistics for inpatients.

The diagnosis of HCC was confirmed in all patients either by histopathological findings or by the appearance of a liver tumor with arterial hypervascularization on contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI) with a serum α-fetoprotein (AFP) value exceeding 400 ng/mL. Liver cirrhosis was diagnosed by histological or clinical features and liver function was evaluated according to the Child-Pugh score. All patients were evaluated according to European Association for the Study of the Liver criteria up to 2005[27], and to American Association for the Study of Liver Diseases criteria from January 2006[28]. We reviewed the patients' records for demographic parameters (age, sex), hepatitis B serology, Child-Pugh stage, AFP, tumor morphology and extension, including maximum diameter, tumor number, and vessel invasion determined by imaging studies (abdominal arteriography, CT during hepatic arteriography, and the portal phase of superior mesenteric arteriography), treatment modality, complications after treatment, hospital mortality, and time of recurrence. Patient characteristics of both survival groups are shown in Table 1.

Treatment

Surgical resection: Surgery was performed with patients under general anesthesia using a right subcostal incision with a midline extension with the aid of intraoperative ultrasound. Anatomic resection was performed using a target resection margin of at least 1 cm. Pringle's maneuver was routinely used with a clamp time of 10 min and an unclamp time of 5 min. Suturing and fibrin glue were used to establish hemostasis on the surface of the liver[29].

TACE: Chemoembolization involves the delivery of chemotherapeutic agents to liver tumors through the hepatic artery. Seldinger's method was used to insert a catheter

Table 1 Baseline characteristics of the patients

Characteristics	5-yr survival group	10-yr survival group	*P* value
No. of patients	1516	520	
Mean age (yr)	50.45 ± 12.32	50.37 ± 11.59	0.102
Female/male patients	166/1350	58/462	0.935
No. of tumors			0.000
1	1285	484	
2	206	33	
⩾ 3	25	2	
No. of treatments			0.000
Surgery	1100	410	
TACE	2356	576	
RFA	656	109	
Mean	2.71 (4112/1516)	2.11 (1095/520)	
Microvascular invasion	7	3	
Histology			0.381
Well	320	104	
Moderate	580	184	
Poor	382	144	
HBsAg			0.104
Positive	948	342	
Negative	568	178	
Cirrhosis			0.000
Absence	1396 (91.1%)	508 (97.7%)	
Present	120 (7.9%)	12 (2.3%)	
Position of lesion			0.246
Left lobe	87	126	
Right lobe	1334	423	
Both lobes	25	5	
Lesion diameter, *n* (cm)			0.651
⩽ 3	214 (2.26 ± 0.62)[1]	82 (2.27 ± 0.69)[1]	
> 3-⩽ 5	404 (4.22 ± 0.60)[1]	143 (4.20 ± 0.61)[1]	
> 5-< 10	560 (12.44 ± 1.98)[1]	190 (7.31 ± 1.28)[1]	
⩾ 10	338 (7.24 ± 1.40)[1]	105 (11.94 ± 1.80)[1]	
Mean	1516 (6.89 ± 3.69)	520 (6.58 ± 3.44)	
Child-Pugh classification			0.383
Class A	1466	505	
Class B	50	15	
TNM stage			0.109
Ⅰ	387	90	
Ⅱ	1096	422	
Ⅲ	33	8	
α-fetoprotein, ng/mL			
⩽ 100	712 (22.50 ± 23.59)[1]	261 (19.78 ± 17.93)[1]	0.528
100-400	186 (257.92 ± 92.89)[1]	48 (204.91 ± 81.92)[1]	0.129
⩾ 400	618 (16661.32 ± 28889.57)[1]	211 (15302.74 ± 23346.16)[1]	0.853
Liver function, mean ± SD			
Total bilirubin, mg/dL	19.65 ± 10.03	21.04 ± 38.16	0.080
AST, U/L	50.82 ± 26.46	51.72 ± 37.24	0.145
ALT, U/L	48.71 ± 27.35	45.32 ± 30.95	0.053
γ-glutamyltransferase, U/L	71.54 ± 43.62	64.42 ± 46.07	0.069

[1]There is significant difference within each group. RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBsAg: Hepatitis B surface antigen.

through the femoral artery. Angiography of the celiac and superior mesenteric arteries was routinely performed to determine the tumor blood supply, distribution of hepatic arteries, and collateral circulation routes[30]. The tumor's primary artery was selected for catheter placement. Patients were given a standard drug regimen of emulsified THP (40-60 mg), DDP (20-60 mg) and lipiodol (5-40 mL) through the hepatic artery.

Radiofrequency ablation: The size and position of the tumors and the position and direction of the needle were confirmed by CT. RFA treatment was performed with patients under general anesthesia to prevent the patient from experiencing pain and to ensure immobilization. CT was used to guide the insertion of a radiofrequency electrode into each tumor. The diameter of the needle was adjusted for tumor size. The range of ablation was extended 0.5-1 cm into the non-cancerous tissue to ensure complete coverage[31]. Patients underwent enhanced CT scans 4 wk after TACE treatment to determine the distribution of lipiodol and the status of any remaining tumor. If living tumor tissue was found, RFA was repeated[32].

Follow-up and recurrence

Dual-phase spiral CT was performed 4 wk after treatment and every 2 mo thereafter for the next 2 years. Each of these follow-up visits included blood tests, including liver function tests and serum AFP tests. Residual viable tumor tissue was considered present upon the first CT assessment at 4 wk after treatment if enhancement areas were seen within the tumor at either the arterial or the portal venous phase. MRI was performed if CT results were unclear on whether residual viable tumor tissue was presentAdditional treatment with RFA was given in these cases. If residual viable tumor was still present after repeated treatments, patients were given TACE[33].

The level of serum AFP and CT scans were regularly assessed to determine tumor recurrence. Recurrence was defined as the presence of hypervascular or early washout tumors on dynamic CT, MRI or angiography, or by a diagnosis of HCC by a radiologist. In this way, the diagnosis of recurrence was based on typical imaging findings on CT or arteriography, and, if necessary, percutaneous fine-needle aspiration cytology. Treatment modalities for HCC included surgery alone, TACE alone, surgery-TACE, TACE-RFA and surgery-TACE-RFA.

Statistical analysis

Patient characteristics are presented as mean ± SD. χ^2 tests and the Wilcoxon rank sum test were used to compare the characteristics of patients in the 5-year and 10-year survival groups. Overall survival was calculated by the Kaplan-Meier method from the beginning of treatment. Death from any cause was considered an event. The differences in survival between groups were compared by the generalized Wilcoxon's test. A stratified Cox's proportional hazards regression model was used for multivariate analysis of prognostic parameters identified by the survival analysis. SPSS statistical software, version SPSS 19.0 for Windows (IBM Corp., Armonk, NY, United States), was used. A P value < 0.05 was considered significant.

RESULTS

Patient characteristics and clinical features

As shown in Table 1, there were 520 patients in the10-

Table 2 Comparison of treatment modalities between 5-year survival group and 10-year survival group for hepatocellular carcinoma

Treatment modalities by tumor type	5-yr survival group	10-yr survival group	*P* value between groups	*P* value between overall groups
No. of treatment modalities				0.092
Surgery alone	609	235		
TACE alone	214	80		
Surgery + TACE	458	125		
TACE + RFA	85	28		
Surgery + TACE + RFA	150	52		
Tumor size ≤ 5 cm (maximum diameter)				0.097
Surgery alone	244 (3.53 ± 1.03)	100 (3.48 ± 1.06)	0.217	
TACE alone	92 (2.59 ± 1.16)	38 (3.58 ± 1.16)	0.087	
Surgery + TACE	133 (3.90 ± 1.02)	43 (3.47 ± 1.03)	0.192	
TACE + RFA	72 (3.10 ± 1.16)	21 (2.93 ± 1.10)	0.824	
Surgery + TACE + RFA	82 (3.88 ± 0.91)	26 (3.42 ± 0.98)	0.314	
Tumor size > 5 cm (maximum diameter)				0.106
Surgery alone	365 (8.16 ± 2.27)	135 (8.532 ± 2.71)	0.130	
TACE alone	122 (9.31 ± 2.94)	42 (9.36 ± 2.26)	0.925	
Surgery + TACE	325 (9.59 ± 3.16)	82 (9.09 ± 2.50)	0.624	
TACE + RFA	13 (11.68 ± 3.74)	7 (10.47 ± 5.59)	0.548	
Surgery + TACE + RFA	68 (7.19 ± 3.02)	26 (10.65 ± 4.63)	0.131	
Uninodular HCC				0.039
Surgery alone	531	223		
TACE alone	178	75		
Surgery + TACE	382	113		
TACE + RFA	57	24		
Surgery + TACE + RFA	100	50		
Multinodular HCC				
Surgery alone	78	12		
TACE alone	36	5		
Surgery + TACE	76	12		
TACE + RFA	28	4		0.445
Surgery + TACE + RFA	50	2		
AFP > 400 ng/mL (AFP value)				0.289
Surgery alone	280 (16053.09 ± 27804.11)	102 (18092.07 ± 26818.62)	0.450	
TACE alone	66 (15876.11 ± 30406.63)	28 (10984.83 ± 18402.28)	0.270	
Surgery + TACE	188 (21164.79 ± 31964.15)	47 (25114.26 ± 31807.02)	0.385	
TACE + RFA	32 (742.50 ± 0.00)	12 (22597.20 ± 28745.64)	0.667	
Surgery + TACE + RFA	56 (1884.33 ± 953.25)	19 (8789.88 ± 21862.41)	0.091	
AFP < 400 ng/mL (AFP value)				0.065
Surgery alone	329 (45.50 ± 71.08)	133 (56.35 ± 91.86)	0.853	
TACE alone	148 (38.60 ± 71.56)	52 (38.09 ± 48.30)	0.355	
Surgery + TACE	270 (66.35 ± 98.86)	78 (43.02 ± 74.45)	0.738	
TACE + RFA	69 (133.34 ± 145.64)	16 (44.81 ± 43.02)	0.579	
Surgery + TACE + RFA	94 (96.06 ± 138.35)	33 (15.43 ± 8.21)	0.064	

AFP: α-fetoprotein; TACE: Transcatheter arterial chemoembolization; RFA: Radiofrequency ablation; HCC: Hepatocellular carcinoma.

year survival group. Single lesions were present in 93.1% and multiple lesions in 6.7%. Lesions located in only one lobe of the liver were present in 93.1%, and lesions located in two lobes were present in 0.9%. A portal vein tumor thrombus was present in 2.3%. Child-Pugh classification class A applied to 97.1% and class B to 2.9%. tumor-node-metastasis (TNM) stage I or Ⅱ applied to 98.5% patients. Among the 1516 patients who survived for 5 years, 84.8% had one lesion, 13.6% had two lesions, 1.6% had three lesions, 98.6% had lesions located in one lobe of the liver, 1.4% had lesions located in the two lobes, 1.1% had vessel invasion, 96.7% had Child-Pugh classification class A, 3.3% had class B, and 97.8% had TNM stage I or Ⅱ. In this way, the survival period of both groups showed some correlation with the number of tumor lesions and liver cirrhosis. No statistically significant differences were observed with respect to maximum tumor diameter in either group. In the 5-year group, vessel invasion appeared in 1.1% of patients. In the 10-year group, vessel invasion appeared in 2.1% of patients. This suggests that patients with vessel invasion seldom survived for 5 years. In the 10-year survival group, 97.1% of patients were described as Child-Pugh classification class A and 2.9% as class B. TNM stage I and Ⅱ was applied to 98.5% of patients. In the 5-year survival group, 96.7% of patients were described as Child-Pugh classification class A, 3.3% as class B, and 97.8% as TNM stage I or Ⅱ. The survival periods of both groups may be related to Child-Pugh classification class A and TNM stage I or Ⅱ.

Treatment modalities

As shown in Table 2, treatment modalities such as surgery alone, TACE alone, surgery-TACE, surgery-TACE-RFA, and TACE-RFA, showed no statistically significant

Table 3 Comparison of treatmentefficacy between the 5-year survival group and 10-year survival group for hepatocellular carcinoma

Characteristics	5-yr survival group	*P* value	10-yr survival group	*P* value	*P* value between groups
AFP the first time before and after treatment (ng/mL)					
Surgery		0.002		0.000	
Before surgery	1020 (4596.63 ± 17193.11)		358 (7419.90 ± 18412.64)		0.049
After surgery	1020 (1133.25 ± 4530.36)		358 (1255.09 ± 4337.24)		0.070
TACE		0.039		0.002	
Before TACE	496 (4510.68 ± 15115.73)		162 (2827.99 ± 9506.04)		0.446
After TACE	496 (1732.58 ± 7631.56)		162 (502.21 ± 1526.13)		0.711
Liver function the first time before and after treatment					
Total bilirubin (mg/dL)		0.721		0.353	
Before treatment	68.32 ± 58.98		19.63 ± 12.65		0.078
After treatment	67.22 ± 69.45		18.76 ± 11.56		0.120
AST (U/L)		0.983		0.730	
Before treatment	51.99 ± 28.41		52.69 ± 40.86		0.137
After treatment	50.87 ± 27.50		52.42 ± 39.77		0.695
ALT (U/L)		0.104		0.262	
Before treatment	52.46 ± 37.21		50.69 ± 53.10		0.291
After treatment	54.60 ± 42.26		51.16 ± 50.09		0.126
γ-glutamyltransferase (U/L)		0.366		0.017	
Before treatment	19.30 ± 8.09		69.85 ± 93.75		0.122
After treatment	19.53 ± 8.32		58.79 ± 67.08		0.670
Time to first tumor recurrence or metastasis (d)		0.012		0.045	
Surgery alone	90 (1031.36 ± 662.61)		38 (3246.38 ± 2047.29)		0.003
TACE alone	32 (675.64 ± 412.31)		12 (1882.77 ± 1324.08)		0.015
Surgery + TACE	225 (1012.00 ± 818.86)		70 (2778.30 ± 6296.35)		0.007
Surgery + TACE + RFA	90 (798.41 ± 492.01)		52 (2553.20 ± 1746.48)		0.016

RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

differences between the 5-year survival group and 10-year survival group, but there were significant differences in treatment modalities of uninodular lesions between the 5-year survival group and 10-year survival group. Surgery alone, TACE alone, surgery-TACE, TACE-RFA and surgery-TACE-RFA showed no statistical differences in the maximum diameter of the tumor for the two groups. The survival period had no relationship with tumor size. Similarly, the survival period had no relationship with the level of the serum AFP in patients treated with different modalities. The patients of the two groups showed statistically significant differences in survival period depending on the different treatment modalities, but only among patients with only one lesion. The patients of the groups showed statistically significant differences in survival period depending on liver cirrhosis. Survival period did not differ significantly across the 5-year survival and 10-year survival groups in patients multiple lesions. In short, different treatment modalities were found to have an effect on patients with only one lesion, and no effect on patients with multiple lesions.

Effects of initial treatment

As shown in Table 3, serum levels of AFP and GGT differed significantly before and after the first treatment, but the levels of serum total bilirubin, aspartate aminotransferase, and alanine aminotransferase showed no statistically significant differences regardless of whether the first treatment was surgery or TACE and regardless of whether the patient survived for 5 or 10 years. The level of pre-surgery serum AFP showed statistically significant differences between 5-year survival and 10-year survival groups. After the first surgery, the two groups showed no statistically significant differences from each other. The time of the first recurrence or metastasis showed significant differences between the 5-year and 10-year survival groups. Different treatments for each treatment group also showed statistically significant differences in the time of the first recurrence or metastasis: (in order of significance) surgery alone > surgery-TACE > TACE alone > surgery-TACE-RFA.

Prognostic analysis

The influence of patient and tumor related factors on overall survival are shown in Table 4. Analysis of patients who survived for 5 years or longer showed that survival rates differed significantly with age, Child-Pugh classification, vessel invasion, liver cirrhosis, TNM stage, treatment method, and number of tumors. Statistical results also showed that, among patients who survived 5 years or longer, no significant differences could be attributed to sex, diameter of the largest tumor, or to pretreatment serum levels of AFP. Analysis of 10 years survival showed statistically significant differences in survival rates with respect to vessel invasion, number of tumors and Child-Pugh classification. No significant differences could be attributed to age, sex, tumor diameter, or pretreatment level of serum AFP. Multivariate analysis of patients who lived 5 years or longer showed survival rates to be related to age, vessel invasion, liver cirrhosis, TNM stage, Child-Pugh classification, treatment method, and number of tumors. Multivariate analysis of patients who survived

Table 4 Comparison of univariate analysis for various prognostic factors between 5-year survival and 10-year survival groups for hepatocellular carcinoma

Factor	5-yr survival group		10-yr survival group	
	Exp (B) 95%CI	*P* value	Exp (B) 95%CI	*P* value
Sex (men and women)	0.787 (0.678-0.913)	0.002	0.875 (0.667-1.146)	0.332
Age (yr) (< 50 and ≥ 50)	0.516 (0.462-0.575)	0.000	1.097 (0.838-1.438)	0.500
Diameter of largest tumor	0.971 (0.921-1.023)	0.274	0.961 (0.879-1.051)	0.384
Child-Pugh classification	0.070 (0.060-0.082)	0.000	0.559 (0.334-0.938)	0.028
No. of tumors	1.452 (1.284-1.642)	0.000	0.390 (0.261-0.582)	0.000
Pretreatment serum AFP level	1.041 (0.987-1.099)	0.138	0.933 (0.851-1.022)	0.137
Cirrhosis	0.832 (0.775-0.893)	0.000	10.539 (8.164-13.606)	0.000
TNM stage	0.739 (0.662-0.862)	0.000	0.911 (0.739-1.124)	0.384
Treatment methods	1.152 (1.109-1.197)	0.000	1.043 (0.975-1.115)	0.220
Microvascular invasion	1.038 (0.494-2.181)	0.922	1.233 (0.396-3.838)	0.718

AFP: α-fetoprotein; TNM: Tumor-node-metastasis.

Table 5 Comparison of multivariate analysis for various prognostic factors between 5-year survival and 10-year survival groups for hepatocellular carcinoma

Group	Sex	Age	Micro-vascular invasion	Pretreatment AFP level	Diameter of tumor	Treatment methods	TNM stage	Child-Pugh classification	No. of tumors	Cirrhosis
5-yr survival (*P* value)	0.344	0.004	0.033	0.080	0.211	0.000	0.001	0.000	0.040	0.000
10-yr survival (*P* value)	0.983	0.962	0.370	0.783	0.847	0.215	0.695	0.029	0.025	0.000

AFP: α-fetoprotein; TNM: Tumor-node-metastasis.

10 years or longer showed survival rates to be related to Child-Pugh classification, liver cirrhosis and to the number of tumors.

Overall survival

Table 1 show that patients with only one lesion, 2 lesions, or 3 lesions showed statistically significant differences with respect to duration of survival period in both groups. Patients who had only one lesion survived significantly longer than other patients. Patients who had 2 lesions seldom survived 5 years. Patients with 3 lesions seldom survived 5 years. Patients who survived more than 5 years were associated with more influencing factors than those in the 10-year survival group. Factors associated with long-term survival among HCC patients included age over 50 years, single rather than multiple lesions, no vessel invasion, TNM stage I or Ⅱ, Child-Pugh classification Class A, and only 1-3 rounds of treatment. Long-term survival of patients for HCC was found to be related to the number of lesions, liver cirrhosis and methods of treatment, not to tumor diameter or level of serum AFP.

Statistical results showed that, among patients who survived 10 years or longer, no significant differences could be obtained from the different treatment modalities (P = 0.202). The survival period of different treatment modalities in the patients who survived between 5 years and 10 years showed statistically significant differences: (in order of significance) surgery alone > surgery-TACE > TACE-RFA > TACE alone > surgery-TACE-RFA (Figure 1). Therefore, the 10-year survival of HCC patients was not associated with the choice of initial treatment modality. However, different treatment modalities had a significant effect on the survival of HCC patients who survived between 5 and 10 years (Table 5).

DISCUSSION

There is a variety of treatment methods and models of HCC. Surgical resection is the preferred treatment for HCC. TACE, however, has broader indications. RFA, microwave ablation, cryoablation, radiation therapy, and high intensity focused ultrasound therapy have been widely used in clinical treatment[34-37]. When HCC is diagnosed early, the curative effects enjoyed by some HCC patients improve, as does the prognosis. However, improvements to the survival rate are very limited and the prognosis remains poor. By evaluating criteria to more accurately predict prognosis, patients at high risk of recurrence would be identified more easily and effective prevention and control measures could be implemented.

The results of this study show the 5-year and 10-year survival groups to have the following common features: lesions located in only one lobe of the liver, single lesions, no vessel invasion, no liver cirrhosis, TNM stage I or Ⅱ, Child-Pugh classification class A, and 1 to 3 treatments. Long-term survival factors are assessed by using univariate and multivariate Cox proportional hazard regression analyses. Multivariate analysis indicated that survival for more than 10 years was associated with treatment modal-

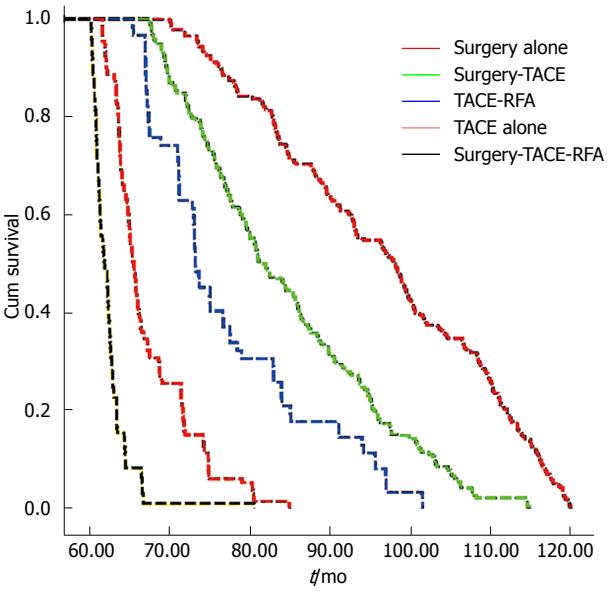

Figure 1 Kaplan-Meier curve shows overall survival rates of different treatment modalities in hepatocellular carcinoma patients who survived between 5 years and 10 years. Different treatment models showed statistically significant differences in the survival period: surgery alone > surgery-transcatheter arterial chemoembolization (TACE) > TACE-radiofrequency ablation (RFA) > TACE alone > surgery-TACE-RFA.

ity, number of lesions, vessel invasion, age, and Child-Pugh classification. Survival for more than 5 years wasassociated with number of lesions, no liver cirrhosis and treatment modalities. The independent prognostic factors of both groups included method of treatment, liver cirrhosis and number of lesions. The diameter of the largest tumor and serum level of AFP were not associated with survival period in either group.

Patients with multiple lesions or lesions located in more than one lobe of the liver showed poorer prognosis and did not always survive 5 years. The treatment modalities showed that the most effective type of treatment was surgery. The three typesof combination therapy were used in 412/520 (79.2%) of the patients who survived for 10 years or longer and in 1244/1516 (82.1%) of the patients who survived for 5 years or longer. The survival period for different treatment modalities in both groups also showed statistically significant differences: surgery alone > surgery-TACE > TACE-RFA > TACE alone > surgery-TACE-RFA. When a single lesion is present and that lesion can be removed by surgery, surgical resection should be the preferred method. Surgical resection is the preferred treatment for HCC. TACE should be performed in the treatment of the relapse or metastatic lesions after surgical resection.

The initial effects of treatment in both 10-year and 5-year survival groups showed serum levels of AFP and GGT to be significantly different before and after the first treatment regardless of whether this first treatment was surgery or TACE. The level of serum AFP showed statistically significant differences between 10-year group and 5-year survival groups before the first surgical operation, but no statistically significant differences between the two groups were detected after the first surgical operation. This suggests that the serum level of AFP decreased quickly after surgical resection in the 10-year survival group, an ideal curative effect. With all five kinds of treatment, surgery alone, surgery-TACE, TACE-RFA, TACE alone, or surgery-TACE-RFA, the first relapse or metastasis showed statistically significant differences between the 5-year and 10-year survival groups. The first relapse or metastasis tended to occur later in the 10-year survival group than in the 5-year survival group. The time to tumor recurrence or metastasis was found to significantly affect the patients' survival periods.

The prognosis of HCC here showed heterogeneity caused by the interactions between many factors and by the interplay between the tumor and the rest of the body. Factors that affect prognosis have been found to be different, but this may be because the studies evaluating them had different goals and factors[38-40]. Lau *et al*[41] reported that the factors that affect the curative effect of mid-to-late HCC are tumor type, portal vein tumor thrombus, treatment method, and hepatic function. Yamamoto *et al*[42] found that portal vein tumor thrombus, tumor type, traces of iodized oil, and hepatic function all influence the survival and prognosis of the HCC patients.

This study evaluated patient age, gender, TNM stage, Child-Pugh classification, portal vein tumor thrombus, serum AFP level, number of tumor lesions, tumor diameter, and treatment method, all of which may have some relationship with prognosis. The independent prognostic survival factors of both the patients who survived more than 10 years and those who survived 5 years can be said to be related to treatment method and to the number of lesions. The number of lesions was found to be a common risk factor in the 10-year and 5-year survival groups. One possible reason for this may be that most of the intrahepatic tumor lesions had undergone intrahepatic metastasis. Multiple lesions tended to be caused by intrahepatic metastasis. Even when tumors were completely removed, subclinical tumor lesions remained in the liver and the tumor cells may have entered the bloodstream. The survival period showed obvious differences in patients who received different treatments. The treatments, in decreasing order of favorability, were as follows: surgery alone > surgery-TACE > TACE-RFA > TACE alone > surgery-TACE-RFA. The patients who survived more than 10 years were found to have more influencing factors than those in the 5-year group. The patients who were more than 50 years old, who had no portal vein tumor thrombus, who had Child-Pugh class A tumors, who had only one lesion, and who underwent appropriate treatment tended to live longer than other patients.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common digestive malignancies cancer, which do serious threat to the people's life and health. Therefore, it has been studied by many scholars, but people still do not know what is the reason for the long-term survival and whether it has an optimal treatment.

Research frontiers

There are various prognosis factors for HCC, some researchers suggesting that age, sex and α-fetoprotein (AFP) level play a critical role, while others suggest that the long-term survival of HCC is related to treatment modality or the biological nature of the tumor.

Innovations and breakthroughs

This manuscript reveals the long-term survival of HCC patients was associated with the number of lesions, liver cirrhosis and Child-Pugh classification. It was not found to be associated with tumor diameter, histological stage, and pretreatment level of serum AFP. Conditions associated with long-term survival of HCC patients were: age over 50 years, no cirrhosis, uninodular lesion, no vessel invasion, tumor-node-metastasis stage Ⅰ or Ⅱ, Child-Pugh classification class A, and appropriate treatment. The clinical reasons for the differences between the 5-year survival and the 10-year survival were found to be the number of lesions, liver cirrhosis, Child-Pugh classification, and time elapsed until first recurrence or metastasis for HCC. The survival period of different treatment modalities in the patients who survived between 5 years and 10 years showed statistically significant differences: surgery alone > surgery-transcatheter arterial chemoembolization (TACE) > TACE-radiofrequency ablation (RFA) > TACE alone > surgery-TACE-RFA. The 10-year survival of HCC patients was not associated with the choice of treatment modality.

Applications

This study retrospectively studied HCC patients treated in the past 20 years, and the ultimate conclusion is that the long-term survival of HCC patients was associated with the number of lesions, liver cirrhosis and Child-Pugh classification, patients who survived for 5 and 10 years showed statistically significant differences in types of treatments. The 10-year survival of HCC patients was not associated with the choice of treatment modality. The results can provide information on the treatment choice of HCC patients.

Terminology

TACE is short for transhepatic arterial chemotherapy and embolization, used in the treatment of HCC patients especially for the terminal cancer patients; RFA is a treatment method for HCC patients which uses a RFA needle to send high radiofrequency waves to heat the tumor and cause cell degeneration and necrosis. It is widely used for small HCC tumor (diameter < 3 cm).

Peer review

This study was performed as a retrospective analysis of prognostic factors including individual therapy for patients with HCC who survived for 5 years or longer and 10 years or longer. A large population of more than 2000 patients was selected from over 8000 HCC patients, and thus this study is likely to give interesting and reliable data.

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BRIEF ARTICLE

Analysis of long non-coding RNA expression profiles in gastric cancer

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Abstract

AIM: To investigate the expression patterns of long non-coding RNAs (lncRNAs) in gastric cancer.

METHODS: Two publicly available human exon arrays for gastric cancer and data for the corresponding normal tissue were downloaded from the Gene Expression Omnibus (GEO). We re-annotated the probes of the human exon arrays and retained the probes uniquely mapping to lncRNAs at the gene level. LncRNA expression profiles were generated by using robust multi-array average method in affymetrix power tools. The normalized data were then analyzed with a Bioconductor package linear models for microarray data and genes with adjusted *P*-values below 0.01 were considered differentially expressed. An independent data set was used to validate the results.

RESULTS: With the computational pipeline established to re-annotate over 6.5 million probes of the Affymetrix Human Exon 1.0 ST array, we identified 136053 probes uniquely mapping to lncRNAs at the gene level. These probes correspond to 9294 lncRNAs, covering nearly 76% of the GENCODE lncRNA data set. By analyzing GSE27342 consisting of 80 paired gastric cancer and normal adjacent tissue samples, we identified 88 lncRNAs that were differentially expressed in gastric cancer, some of which have been reported to play a role in cancer, such as LINC00152, taurine upregulated 1, urothelial cancer associated 1, Pvt1 oncogene, small nucleolar RNA host gene 1 and LINC00261. In the validation data set GSE33335, 59% of these differentially expressed lncRNAs showed significant expression changes (adjusted *P*-value < 0.01) with the same direction.

CONCLUSION: We identified a set of lncRNAs differentially expressed in gastric cancer, providing useful information for discovery of new biomarkers and therapeutic targets in gastric cancer.

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Key words: Long non-coding RNA; Gastric cancer; Microarray analysis; Data mining

Core tip: Long non-coding RNAs (lncRNAs) have risen to prominence with important roles in a broad range of biological processes. LncRNA expression patterns and their biological functions in gastric cancer still remain unknown. We re-annotated the probes from an Affymetrix Human Exon 1.0 ST array and identified probes uniquely mapping to lncRNAs at the gene level. These probes correspond to 9294 lncRNAs, covering nearly 76% of the GENCODE lncRNA data set. We identified a set of lncRNAs that were differentially expressed in gastric cancer. In an independent data set, 59% of these differentially expressed lncRNAs showed significant expression changes with the same direction.

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non-coding RNA expression profiles in gastric cancer. *World J Gastroenterol* 2013; 19(23): 3658-3664 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3658.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3658

INTRODUCTION

Over the last decade, advances in genome-wide analysis of gene expression have revealed far more genomic transcription than previously anticipated, with the majority of the genome being transcribed into non-coding RNAs (ncRNAs)[1,2]. Much attention has focused on microRNAs (miRNAs), one class of small non-coding RNAs. MiRNAs are involved in specific regulation of both protein-coding and putatively non-coding genes by post-transcriptional silencing or infrequently by activation[3-5].

More recently, long non-coding RNAs (lncRNAs), generally defined as having a size greater than 200 nucleotides, have risen to prominence with important roles in a broad range of biological processes. LncRNAs regulate gene expression at the level of post-transcriptional processing such as protein synthesis, RNA maturation, and transport. They also exert their effects in transcriptional gene silencing through the regulation of chromatin structure[6,7]. Dysregulation of lncRNAs is associated with many human diseases, including various types of cancers[8]. The well-studied lncRNA HOTAIR, for example, was found to be highly upregulated in both primary and metastatic breast tumors, and its expression level in primary tumors was a powerful predictor of eventual metastasis and death[9]. However, lncRNA expression patterns and their biological function in gastric cancer remain unknown.

In this study, we identified a set of lncRNAs that were differentially expressed in gastric cancer by analyzing publicly available data sets from the gene expression omnibus (GEO).

MATERIALS AND METHODS

Microarray data

Human exon arrays for gastric cancer and normal adjacent tissue were downloaded from the GEO. Two data sets were included: GSE27342 and GSE33335. GSE27342 consisted of 80 paired gastric cancer and normal adjacent tissue, including 4 stage Ⅰ, 7 stage Ⅱ, 54 stage Ⅲ and 7 stage Ⅳ[10,11]. All samples were taken from three hospitals affiliated with Jilin University College of Medicine and Jilin Provincial Cancer Hospital, Changchun, China. GSE33335 consisted of 25 paired gastric cancer and normal adjacent tissue obtained from the tissue bank of Shanghai Biochip Center, Shanghai, China[12,13]. Three raw CEL files failed to be normalized and were excluded from our analysis, leaving 22 paired gastric cancer and normal adjacent tissue. GSE27342 was used as an experimental set to discover differentially expressed lncRNAs in gastric cancer while GSE33335 was used as a validation set.

Probe re-annotation pipeline

The sequences of protein-coding transcripts were retrieved from Ensembl release 67[14], UCSC[15] and RefSeq release 54[16] in July 2012. Specifically, the protein-coding transcripts are a pool of transcripts with gene_type as "protein_coding" in Ensembl, transcripts with category as "coding" in UCSC and transcripts with an identifier beginning with NM_ in RefSeq. The sequences of non-coding transcripts were compiled from Ensembl through Biomart. The probe sequences of the human exon array were downloaded from the Affymetrix website (http://www.affymetrix.com/Auth/analysis/downloads/na25/wtexon/HuEx-1_0-st-v2.probe.tab.zip) and aligned to the sequences of protein-coding and non-coding transcripts using BLAST-2.2.26+[17]. The alignment results were then filtered by the following steps: (1) probes perfectly matched to a transcript were retained; (2) probes mapped to non-coding transcripts only were retained; (3) probes mapped to unique genes were retained; (4) probes mapped to known lncRNAs (genes annotated with processed_transcript, lincRNA, antisense, non_coding, sense_intronic, ncrna_host, sense_overlapping and 3prime_overlapping_ncrna) were retained; and (5) genes with less than 3 probes were removed.

A new PGF file covering the re-annotated probe-lncRNA relationships was created. The official CLF file HuEx-1_0-st-v2.r2.clf was downloaded from the Affymetrix website (http://www.affymetrix.com/Auth/support/downloads/library_files/HuEx-1_0-st-v2.r2.zip).

Data analysis

Gene expression profiles were summarized by applying robust multi-array average (RMA)[18] normalization as implemented in affymetrix power tools (1.14.4 package apt-probeset-summarize), using the newly-created PGF file and the official CLF file.

The normalized data were analyzed with a Bioconductor package linear models for microarray data (LIMMA), a modified *t*-test incorporating the Benjamini-Hochberg multiple hypotheses correction technique[19]. Genes with adjusted *P*-values below 0.01 were considered differentially expressed. The heatmap of differentially expressed genes was generated using BRB-Array Tools Version 4.3.0 Beta 1 (http://linus.nci.nih.gov/BRB-ArrayTools.html)[20].

RESULTS

Re-annotation of exon array probes

A computational pipeline was established to re-annotate over 6.5 million probes of the Affymetrix Human Exon 1.0 ST array (Figure 1). There were 315255 probes perfectly matched to non-coding RNAs but not to any protein-coding transcript. These probes were mapped from transcript level to gene level and 278918 probes matched to one gene were retained. Probes mapping to

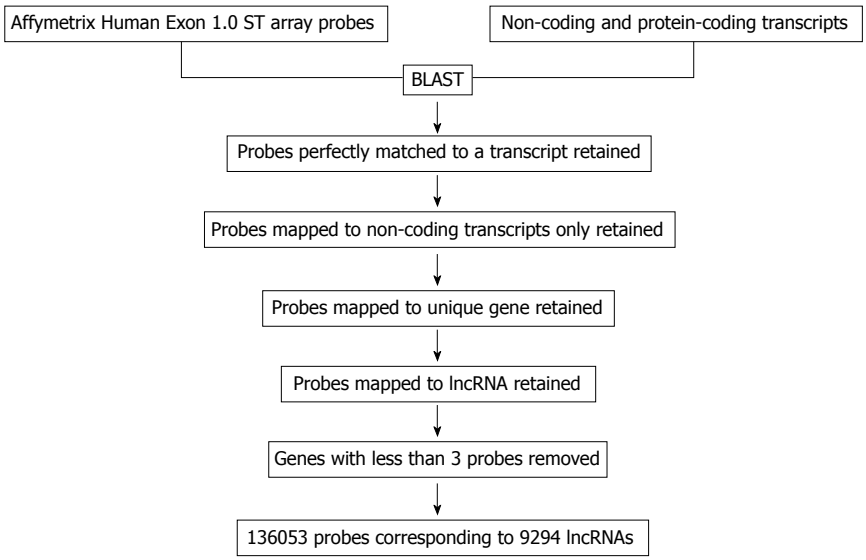

Figure 1 Computational pipeline for re-annotating the probes of the Affymetrix Human Exon 1.0 ST array. lncRNA: Long non-coding RNA.

short ncRNAs and pseudogenes were then discarded, leaving 136,533 probes mapping to lncRNAs, which were annotated with Ensembl (processed_transcript, lincRNA, antisense, non_coding, sense_intronic, ncrna_host, sense_overlapping and 3prime_overlapping_ncrna). To further increase accuracy, genes matched by less than three probes were discarded. Finally, we obtained 136053 probes uniquely mapping to lncRNAs at the gene level, corresponding to 9294 lncRNAs. The number of probes mapping to lncRNAs ranged from 3 to 257 and the average was 18.

Identification of differentially expressed lncRNAs in gastric cancer

The CEL files were processed by Affymetrix Power Tools for background correction, normalization, and summarizations with RMA algorithm. Using LIMMA with an adjusted *P*-value of less than 0.01 as a threshold, we identified 88 lncRNAs that were differentially expressed in gastric cancer as compared to normal gastric tissue (Figure 2). The top 30 lncRNAs differentially expressed in gastric cancer are listed in Table 1. Of 88 differentially expressed lncRNAs, 71 lncRNAs were found to be upregulated and 17 to be downregulated. Most of these lncRNAs do not have an official Human Genome Nomenclature Committee symbol and their function is unknown. But some have been reported to play a role in cancer, including LINC00152[21], taurine upregulated 1 (TUG1)[22], urothelial cancer associated 1 (UCA1)[23,24], Pvt1 oncogene (PVT1)[25], small nucleolar RNA host gene 1 (SNHG1)[26], and LINC00261[27].

Validation in an independent data set

To independently validate our results, we conducted the same analysis on GSE33335 and found that 59% of the differentially expressed lncRNAs identified by above analysis showed significant expression changes (adjusted $P < 0.01$) with the same direction. As shown in Figure 3, the distribution of expression differentials between the experimental data set and the validation data set is significantly concordant, reflecting a high consistence in expression patterns of these genes among different sample sets.

DISCUSSION

LncRNAs have comprehensive functions in biological processes through various mechanisms[6,7]. The expression patterns of lncRNAs are of great importance in the cancer field and are often investigated with tiling arrays[28,29], RNA sequencing[30] or lncRNA-specific microarrays[31,32], which are relatively expensive and inflexible. Recently, studies have suggested that lncRNA expression profiling may be achieved by mining existing microarray data because some probes uniquely mapping to lncRNAs are fortuitously represented on these arrays[33,34]. The Affymetrix Human Exon 1.0 ST array consists of over 6.5 million individual probes designed along the entire length of the gene as opposed to just the 3' end, providing a unique platform for mining lncRNA profiles[35,36].

The lncRNA list used in this study was retrieved from Ensembl and is equivalent to the GENCODE lncRNA data set. This data set utilizes a combination of manual curation, computational analysis and targeted experimental approaches, and is the largest catalog of human lncRNAs to date[22]. To filter out potentially unrecognized probes mapping to protein-coding genes, we generated a merged known protein-coding gene list from RefSeq, UCSC and Ensembl. Still, some probes could potentially hybridize to other undiscovered transcripts or genes.

We identified 136053 probes from the Affymetrix Human Exon 1.0 ST array uniquely mapping to lncRNAs at the gene level. These probes correspond to 9294 lncRNAs, covering nearly 76% of the GENCODE lncRNA data set. This analysis revealed a set of lncRNAs that were differentially expressed between gastric cancer and normal gastric tissue, some of which have been pre-

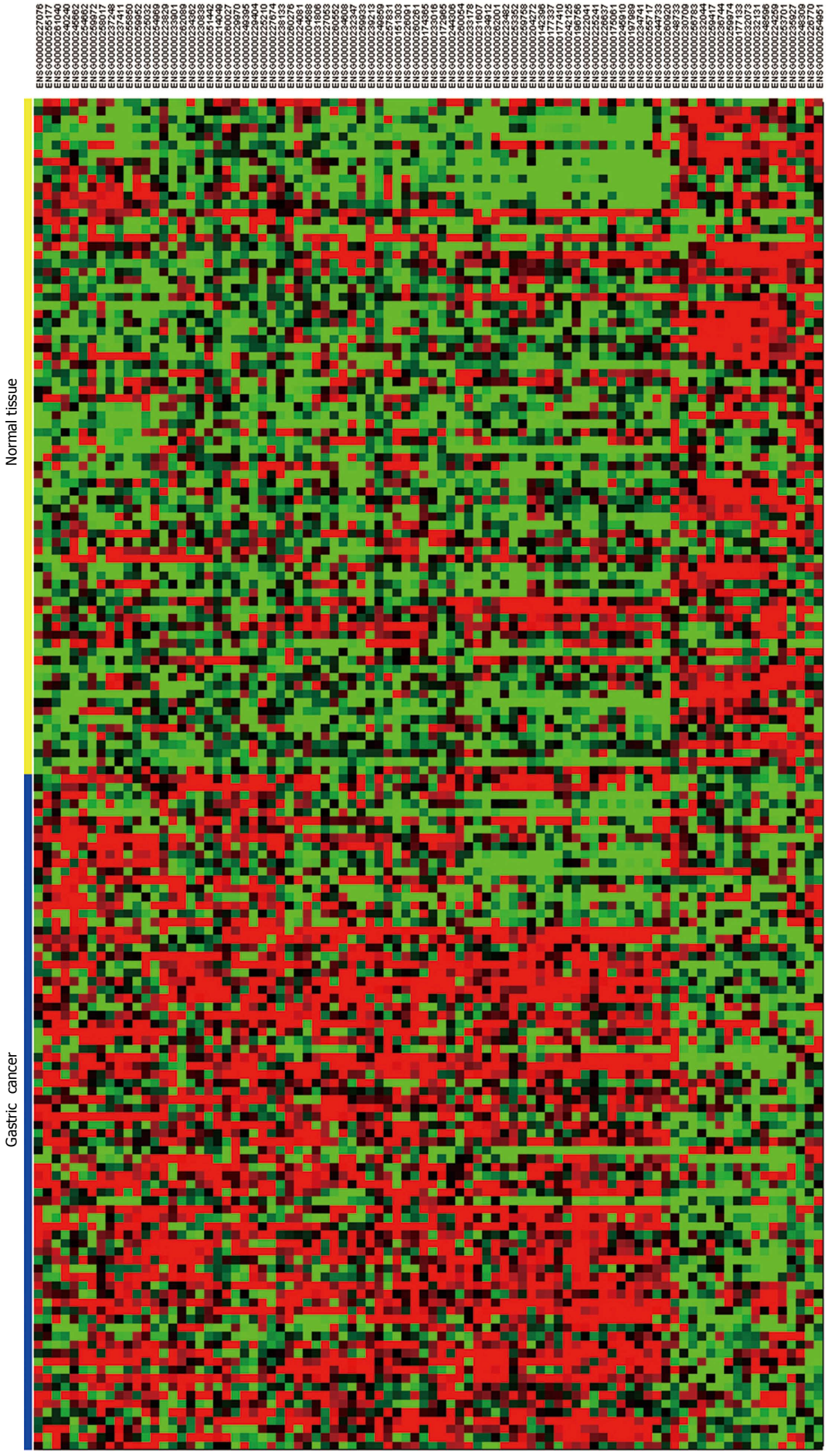

Figure 2 Clustering heatmap of 80 paired samples based on the 88 differentially expressed long non-coding RNAs. Each column represents one sample and each row represents one long non-coding RNA. Gene expression levels are indicated as follows: red, high expression; green, low expression.

Table 1 Top 30 long non-coding RNAs differentially expressed in gastric cancer

Ensembl gene ID	Fold change	Adjusted *P*-value	Gene biotype	HGNC symbol
ENSG00000222041	1.92678	6.89E-09	lincRNA	LINC00152
ENSG00000177410	1.54450	2.32E-06	lincRNA	ZNFX1-AS1
ENSG00000242125	1.65582	9.09E-06	processed_transcript	SNHG3
ENSG00000196756	1.31942	1.26E-05	lincRNA	
ENSG00000255717	1.49039	2.30E-05	ncrna_host	SNHG1
ENSG00000226637	1.43434	2.30E-05	lincRNA	
ENSG00000177337	1.40114	2.30E-05	lincRNA	
ENSG00000249859	1.35920	2.98E-05	lincRNA	PVT1
ENSG00000197989	1.20284	6.10E-05	lincRNA	SNHG12
ENSG00000172965	1.23947	7.60E-05	processed_transcript	
ENSG00000234741	1.37852	9.11E-05	non_coding	GAS5
ENSG00000177133	0.79465	9.11E-05	processed_transcript	
ENSG00000255850	1.38946	1.45E-04	antisense	
ENSG00000232131	0.69562	1.77E-04	antisense	
ENSG00000244306	1.20990	2.64E-04	lincRNA	
ENSG00000234608	1.20458	2.72E-04	lincRNA	C12orf47
ENSG00000259758	1.37499	2.72E-04	antisense	
ENSG00000262001	1.24714	2.72E-04	lincRNA	
ENSG00000259974	0.53383	3.56E-04	lincRNA	LINC00261
ENSG00000237248	1.32118	3.89E-04	lincRNA	
ENSG00000249395	1.49799	4.04E-04	lincRNA	
ENSG00000175061	1.21582	4.68E-04	non_coding	C17orf76-AS1
ENSG00000253352	1.39461	4.68E-04	lincRNA	TUG1
ENSG00000260920	1.46802	5.75E-04	sense_overlapping	
ENSG00000248309	0.88939	5.94E-04	lincRNA	
ENSG00000231806	1.36155	6.48E-04	lincRNA	
ENSG00000223482	1.23001	6.48E-04	antisense	
ENSG00000227076	1.22709	6.48E-04	sense_intronic	
ENSG00000225241	1.20664	6.48E-04	lincRNA	NBPF8
ENSG00000236744	0.74935	6.48E-04	processed_transcript	

HGNC: Human Genome Nomenclature Committee; TUG1: Taurine upregulated 1; SNHG: Small nucleolar RNA host gene.

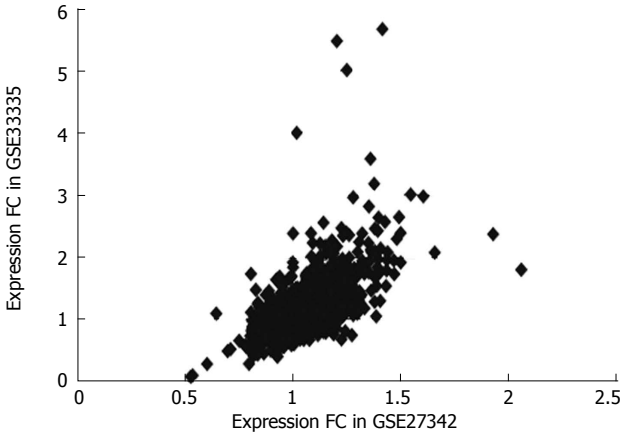

Figure 3 Distribution of expression differentials between experimental data set GSE27342 and validation data set GSE33335.

viously reported in human cancers. For example, TUG1 is upregulated in bladder urothelial carcinoma and high TUG1 expression levels were associated with high grade and stage carcinomas[22]. Knockdown of TUG1 induced cell proliferation inhibition and apoptosis. Another candidate, UCA1, is dramatically upregulated in bladder cancer, suggesting it may be a very sensitive marker for bladder cancer[23]. Exogenous expression of UCA1 enhanced tumorigenicity, invasive potential, and drug resistance in BLS-211 cells[24]. Also, PVT1, located in 8q24 and amplified and overexpressed in ovarian and breast cancer, increases cell proliferation and inhibits apoptosis[25]. In light of published results in other cancers, we hypothesize that these lncRNAs may play an important role in the development of gastric cancer and are potential candidates for new biomarkers and therapeutic targets in gastric cancer.

Recently, H19 was shown to be upregulated in gastric cancer and its overexpression contributes to proliferation of gastric cancer cells[37]. In our study, the fold change of H19 in gastric cancer versus normal tissue is 1.378 with an adjusted *P*-value of 0.043. Though the difference was not statistically significant with our threshold (an adjusted *P*-value less than 0.001), the trend is consistent with the early report. However, our analysis may miss some lncRNAs that other groups have demonstrated to be involved in the development of gastric cancer due to the different distributions of the patient populations in terms of age, gender and cancer subtype and stage. We are also interested in exploring which lncRNAs are differentially expressed in different stages of gastric cancer. Unfortunately, the majority of gastric cancer samples in GSE27342 are stage Ⅲ (54/72), making it is challenging to identify differentially expressed lncRNAs based on the stage of disease.

In conclusion, we presented global lncRNA expression profiles in gastric cancer by mining existing microar-

ray data sets. We identified a set of lncRNAs that were differentially expressed in gastric cancer, revealing potential candidates for gastric cancer biomarkers, potentially improving diagnosis and therapy.

COMMENTS

Background

Long non-coding RNAs (lncRNAs) are an important class of regulatory transcripts involved in a variety of biological functions. While they are aberrantly expressed in many types of cancers, their expression patterns and biological functions in gastric cancer remain unknown.

Research frontiers

LncRNA expression profiles are often investigated with tiling arrays, RNA sequencing or lncRNA-specific microarrays. Existing microarray data represent unique probes specific to lncRNAs, suggesting lncRNA expression profiling may be achieved by mining existing microarray data.

Innovations and breakthroughs

The authors re-annotated the probes of the Affymetrix Human Exon 1.0 ST array and identified probes uniquely mapping to lncRNAs at the gene level. By analyzing a publicly available data set, a set of lncRNAs differentially expressed in gastric cancer were identified.

Applications

The study results suggest lncRNAs play an important role in the development of gastric cancer and have the potential to be used as molecular diagnostic markers and therapeutic targets in gastric cancer.

Terminology

LncRNAs are non-protein coding transcripts having a size greater than 200 nucleotides. This limit distinguishes lncRNAs from small non-coding RNAs such as microRNAs, short interfering RNAs, Piwi-interacting RNAs, and small nucleolar RNAs.

Peer review

The authors investigated the expression patterns of lncRNAs in gastric cancer by mining existing microarray data sets. A set of lncRNAs differentially expressed in gastric cancer were identified. These results are interesting and suggest that lncRNAs may play an important role in gastric cancer.

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BRIEF ARTICLE

Double-balloon enteroscopy in small bowel tumors: A Chinese single-center study

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Abstract

AIM: To analyze the clinical characteristics of small bowel tumors detected by double-balloon enteroscopy (DBE) and to evaluate the diagnostic value of DBE in tumors.

METHODS: Four hundred and forty consecutive DBE examinations were performed in 400 patients (250 males and 150 females, mean age 46.9 ± 16.3 years, range 14-86 years) between January 2007 and April 2012. Of these, 252 patients underwent the antegrade approach, and 188 patients underwent the retrograde approach. All the patients enrolled in our study were suspected of having small bowel diseases with a negative etiological diagnosis following other routine examinations, such as upper and lower gastrointestinal endoscopy and radiography tests. Data on tumors, such as clinical information, endoscopic findings and operation results, were retrospectively collected.

RESULTS: Small bowel tumors were diagnosed in 78 patients, of whom 67 were diagnosed using DBE, resulting in a diagnostic yield of 16.8% (67/400); the other 11 patients had negative DBE findings and were diagnosed through surgery or capsule endoscopy. Adenocarcinoma (29.5%, 23/78), gastrointestinal stromal tumor (24.4%, 19/78) and lymphoma (15.4%, 12/78) were the most common tumors. Among the 78 tumors, 60.3% (47/78) were located in the jejunum, and the overall number of malignant tumors was 74.4% (58/78). DBE examinations were frequently performed in patients with obscure gastrointestinal bleeding (47.4%) and abdominal pain (24.4%). The positive detection rate for DBE in the 78 patients with small bowel tumors was 85.9% (67/78), which was higher than that of a computed tomography scan (72.9%, 51/70). Based on the operation results, the accuracy rates of DBE for locating small bowel neoplasms, such as adenocarcinoma, gastrointestinal stromal tumor and lymphoma, were 94.4%, 100% and 100%, respectively. The positive biopsy rates for adenocarcinoma and lymphoma were 71.4% and 60%, respectively.

CONCLUSION: DBE is a useful diagnostic tool with high clinical practice value and should be considered the gold standard for the investigation of small bowel tumors.

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Key words: Double-balloon enteroscopy; Small bowel tumors; Diagnosis; Capsule endoscopy; Endoscopic findings

Core tip: This was a single-center study with a large sample size of patients who underwent 440 consecutive double-balloon enteroscopy (DBE) examinations. The detection rates of various tumors, location of the

lesions, histological analyses and reasons for DBE were evaluated. Differences in the rates of detecting small bowel tumors between abdominal computed tomography, capsule endoscopy and DBE were compared. Based on the operation results, we analyzed the accuracy of DBE for locating neoplasms in addition to its positive biopsy rate. DBE's high clinical practice value indicated that it should be considered as the gold standard for small bowel tumors.

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INTRODUCTION

Long considered to be a "black box" in the GI tract, the small bowel has been inaccessible to the endoscopist because of its anatomy, location and tortuosity. Small bowel tumors are relatively rare disorders and account for 3%-6% of all digestive neoplasms, most of which are malignant, and represent only 1.1%-2.4% of gastrointestinal malignancies[1]. This low incidence may be ascribed to its unique physiological features, which include an alkaline environment, fluidity, low bacterial count and a high level of IgA in the small intestine[2]. The diagnosis and management of small bowel tumors are formidable tasks for physicians.

The advent of capsule endoscopy (CE) and double-balloon enteroscopy (DBE) has completely changed our approach and launched a new era for small bowel diseases. DBE is a relatively noninvasive method, with a diagnostic yield of approximately 43%-80%[3,4]. Total enteroscopy can be achieved through antegrade and retrograde procedures. Compared with the use of CE in small bowel tumors, DBE has the particular advantage of biopsy and therapeutic potential, such as endoscopic stenting, balloon dilatation and localization before operation[5]. Additionally, DBE is not contraindicated in patients with stenosis of the intestine or an obstruction caused by neoplasms.

From January 2007 to April 2012, 440 examinations were performed in 400 patients, of whom 78 were diagnosed with small bowel tumors. However, little data involving large patient samples are available regarding the diagnostic value of DBE for small bowel tumors in China. In this context, our study was conducted to determine the characteristics of small bowel tumors in patients undergoing DBE and to evaluate the clinical value of DBE.

MATERIALS AND METHODS

Patients

A retrospective, descriptive study involving all patients who were admitted to our hospital for DBE from January 2007 to April 2012 was conducted. Four hundred patients were enrolled in the present series (250 males and 150 females with a mean age of 46.9 ± 16.3 years, range 14-86 years). The indications included the following: obscure gastrointestinal bleeding (OGIB) in 149 cases, abdominal pain in 123 cases, chronic diarrhea in 40 cases, The other 88 cases involved weight loss, abnormalities on computed tomography (CT) scan or CE, and anemia. The main characteristics of the patients are shown in Table 1. All the patients were suspected of having small bowel diseases, and other routine examinations, such as gastroscopy, colonoscopy, abdomen CT, and radiography, did not reveal an etiological diagnosis. The data collected included age, sex, the indication for DBE, the insertion length, the diagnosis and the results of the operation.

DBE system and procedure

All DBE examinations were performed with a Fujinon enteroscope (EN450-P5/20, Fujinon, Inc, Saitama, Japan). The operating system consisted of a mainframe, an enteroscope, an overtube and an air pump. Two soft latex balloons, which could be inflated and deflated, were attached to the tip of the enteroscope and overtube. The balloons were connected to a pump that modulated the air automatically through an air channel in the endoscope, according to the different balloon pressures required. To reduce friction between the enteroscope and the overtube, olive oil and water were added as lubricants to the lacuna between them during the operation. When the procedure was performed as described by Yamamoto[6], the endoscope achieved deep advancement into the small bowel using the overtube in coordination with the serial inflation and deflation of the balloons.

DBE was performed *via* the oral, anal or both approaches at the discretion of the endoscopist and according to the presumed location of the suspected lesions. When the location was uncertain, the oral approach was preferred.

Preoperative preparation

For both the antegrade and retrograde approaches, preparation included overnight fasting and the consumption of three boxes of polyethylene glycol electrolyte (69.56 g × 3) diluted in 3000 mL of water 5-6 h before the examination.

DBE was carried out under conscious or deep sedation when required. Sedation was achieved with the help of an anesthesiologist. Conscious sedation required the intravenous injection of midazolam and meperidine. General anesthesia was indicated for select patients who were administered a combination of propofol and fentanyl. Patients who underwent DBE *via* the oral approach with deep sedation requested a tracheal cannula. The cardiovascular risk status of the patients was evaluated before the examination. During DBE, oxygen was administered along with electrocardiographic monitoring when necessary.

Table 1 Patient characteristics *n* (%)

Characteristic	*n* = 400
Age, yr, median (range)	46.9 ± 16.3 (14-86)
Sex (male/female)	250/150
Reasons for DBE	
OGIB	149 (37.3)
Abdominal pain	123 (30.7)
Chronic diarrhea	40 (10.0)
Others	88 (22.0)
Tumors detected by DBE	*n* = 78
OGIB	37 (47.4)
Abdominal pain	19 (24.4)
Intestinal obstruction	8 (10.3)
Others	14 (17.9)

DBE: Double-balloon enteroscopy; OGIB: Obscure gastrointestinal bleeding.

Table 2 Tumors detected in the present study

Tumor	DBE (positive/negative)	Detection rate	Benign/malignant	Duodenum /jejunum/ileum
Adenocarcinoma	23 (22/1)	29.50%	0/23	6/16/1
GIST	19 (16/3)	24.40%	6/13	3/12/4
Lymphoma	12 (9/3)	15.40%	0/12	1/6/5
Lipoma	8 (7/1)	10.30%	8/0	0/8/0
Metastatic carcinoma	8 (8/0)	10.30%	0/8	3/2/3
Hamartoma	2 (2/0)	2.60%	2/0	1/0/1
Others[1]	6 (3/3)	7.70%	4/2	2/3/1
Total	78 (67/11)	100%	20/58	16/47/15

[1]1 duodenal Brunner's adenoma, 1 ileal hemangioma, 1 duodenal gangliocytic paraganglioma, 1 jejunal mesangial fibrosarcoma,1 jejunal inflammatory myofibroblastic tumor, 1 jejunal serosa fibromatosis. DBE: Double-balloon enteroscopy; GIST: Gastrointestinal stromal tumor.

Statistical analysis

The SPSS 16.0 software package was used for statistical analysis. Count data were expressed as a percentage, and measurement data were expressed as the mean ± SD. Differences were evaluated with the χ^2 test. We used Fisher's exact probability when the theoretical frequency was less than 5. $P < 0.05$ (two sided) was considered to be statistically significant.

RESULTS

In this study, 440 DBE procedures were performed in 400 patients (252 antegrade, 188 retrograde); 40 patients underwent both antegrade and retrograde procedures. Two patients completed the entire small intestine examination all at once *via* the oral approach. Seventy-eight cases of small bowel tumors were detected, giving a positive rate of 19.5% (78/400). Clinically positive DBE findings were observed in 67 patients.

All procedures were successfully performed, except for three patients who had a perforation after the examination. No hemorrhage, acute pancreatitis or other serious complications occurred. Uncomfortable feelings, such as nausea, abdominal distension and abdominal pain, occurred in some cases during the examination. However, these symptoms were transient and tolerable. The complication rate was 0.68% (3/440) in our study group.

Tumors detected in our study

More than 10 types of tumors (Table 2) were found in our study. The majority of these tumors were adenocarcinoma, followed by gastrointestinal stromal tumor (GIST) and lymphoma. In contrast, some tumors had a low detection rate, such as lipoma, metastatic carcinoma and hamartoma. Some extremely rare cases, such as duodenal gangliocytic paraganglioma, jejunal mesangial fibrosarcoma, inflammatory myofibroblastic tumor and serosa fibromatosis, were also found in our group. The typical endoscopic images of the main tumors are shown in Figure 1.

In the 11 patients with negative DBE results, tumors were detected through surgery or capsule endoscopy and included three lymphomas, three GIST, one adenocarcinoma and one lipoma (Table 2). The reasons for the missed diagnoses were as follows: the depth of insertion was inadequate (five cases), the choice of insertion approach was not optimal and the tumors were located at the opposite end of the intestine (four cases), and the tumors were exophytic growths with normal intestinal mucosa (two cases).

Delineation results

Location of lesions: In general, we determined the approximate location through the inserted depth of the endoscope, the size of the enteric cavity, and the shape of the mucosal fold and villi. Among the 78 tumors identified in the patients, those located in the jejunum had the highest detection rate (60.3%, 47/78). The detection rate of tumors in the duodenum was 20.5% (16/78), which was similar to that of tumors located in the ileum (19.2%). Most of the tumors, such as adenocarcinoma, lymphoma, GIST and lipoma, had a high incidence rate in the jejunum.

Histological analysis: Malignant tumors were found in 58 patients, with a detection rate of 74.4% (58/78). The distribution of tumors was as follows: 23 adenocarcinoma, 13 malignant GIST, 12 lymphoma, eight metastatic carcinoma and two others. In our study, compared with malignant tumors, benign tumors had lower detection rates (25.6%, 20/78); the primary benign tumors were eight lipomas and six GIST.

Reasons for DBE: Of the 78 patients with small bowel tumors, OGIB was the most common reason for DBE, followed by abdominal pain, intestinal obstruction and others, which included abdominal distention, vomiting, diarrhea and weight loss (Table 1).

Comparisons between DBE and other imaging modalities

The positive detection rate for DBE in the 78 patients

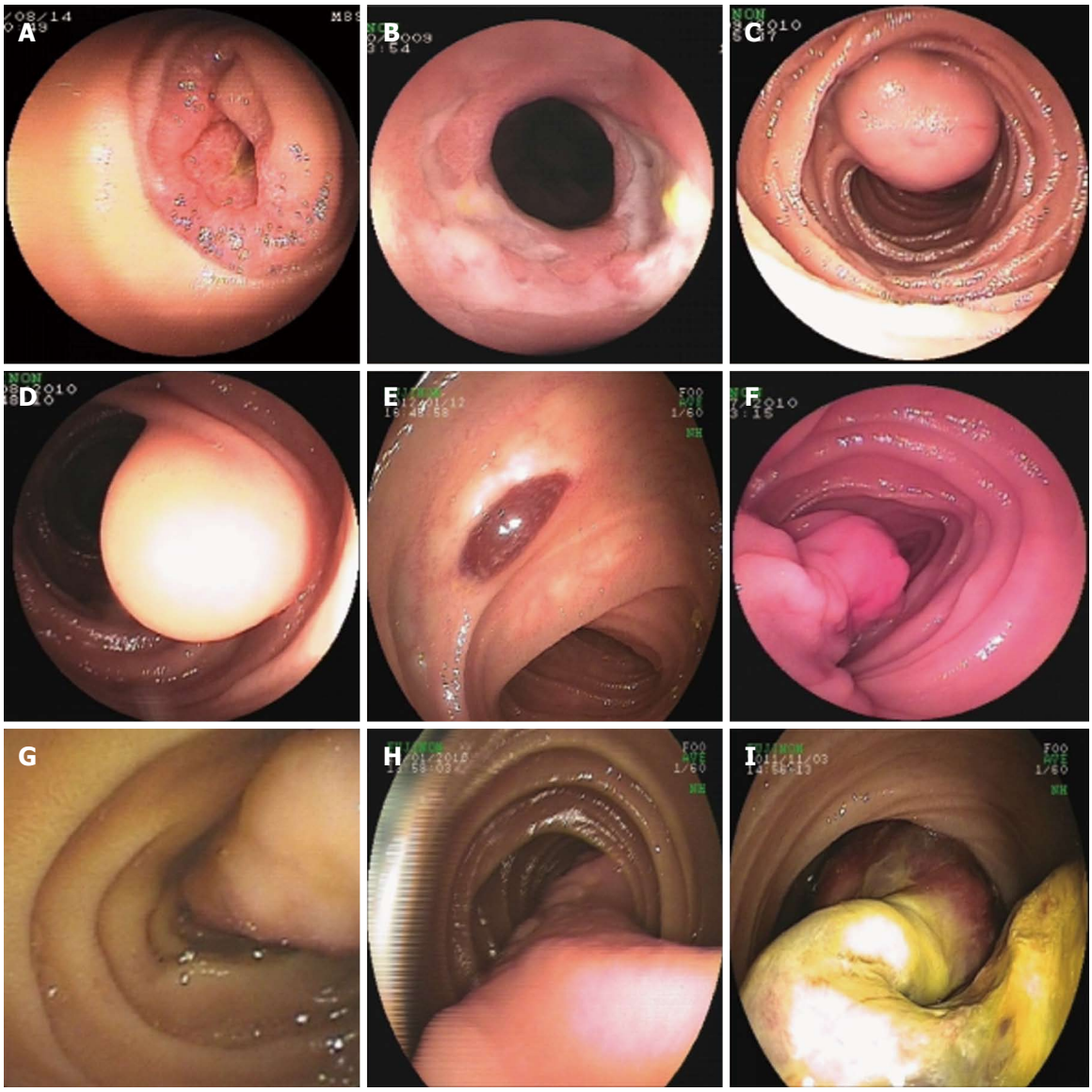

Figure 1 Typical gastrointestinal imaging. A: Adenocarcinoma; B: Lymphoma; C: Gastrointestinal stromal tumor; D: Lipoma; E: Hemangioma; F: Gangliocytic paraganglioma; G: Brunner's adenoma; H and I: Hamartoma.

with small bowel tumors was 85.9% (67/78). Seventy of those 78 cases underwent an abdominal CT. If the CT showed a small mural mass, wall thickening with enhancement or luminal narrowing, then the results were considered positive. The positive rate for CT was 72.9% (51/70). CE was used to examine 27 cases, with a positive rate of 77.8% (21/27). Twenty-two patients underwent CE examinations before DBE; only five cases had the DBE examination first. We performed a statistical analysis of the detection rates for DBE, CE and abdominal CT. The results indicated that DBE had a higher detection rate compared with CT ($P < 0.05$). There were no significant differences between DBE and CE or CE and CT ($P > 0.05$).

Operation results

In general, we determined the types of tumors through the endoscopic features, imaging results and other auxiliary examinations performed before the operation. An endoscopic biopsy was carried out for some tumors. Fifty-three cases (68.0%) underwent an operation in this series of patients.

As a result of the operation, 18 cases were confirmed to have an adenocarcinoma, which was the most common tumor type in this group, followed by GIST (14 cases) and lymphoma (8 cases); the other 13 cases included metastatic carcinoma and hamartoma. Of the 53 patients who underwent an operation in our study, the positive rate of DBE in the 18 cases of adenocarcinoma was 100% (18/18), whereas the positive rate was 78.6% (11/14) for GIST and 62.5% (5/8) for lymphoma. The accuracy rates of DBE in locating small bowel neoplasms, such as adenocarcinoma, GIST and lymphoma, were 94.4%, 100% and 100%, respectively. The positive biopsy rates for adenocarcinoma and lymphoma were 71.4% and 60%, respectively.

DISCUSSION

Although the small intestine comprises nearly 75% of the GI tract extension and nearly 90% of its mucosal surface, small bowel neoplasms are rare[7]. Recent epidemiological studies have indicated that the incidence of small bowel neoplasms has increased, particularly for malignant tumors, because of changes in diet and lifestyle[8]. Currently, the development of CE and DBE has made endoscopic examination of the entire small bowel practical. Although CE examination has revolutionized the standard small bowel evaluation[9], some technical limitations hamper its potential usefulness and effectiveness, including its inability to sample tissue and perform therapeutic procedures. These drawbacks have been overcome with the introduction of DBE, which allows dynamic observation with the controlled movement of the endoscope, the collection of biopsies and many types of interventional procedures[10]. Under some circumstances, it can be assumed that most investigational laparotomies will be replaced by DBE.

Among the 400 patients who were submitted to examination with DBE, 67 of the 78 patients (78/400, 16.8%) who were eventually found to have small bowel tumors were detected with DBE. The results revealed that adenocarcinoma, GIST and lymphoma were the three most common tumors. In our study, adenocarcinoma had the highest incidence among the malignant neoplasms, while lipoma was the most common benign tumor. Some extremely rare tumors reported only in single cases[11,12], including duodenal gangliocytic paraganglioma, jejunal mesangial fibrosarcoma, jejunal inflammatory myofibroblastic tumor and jejunal serosa fibromatosis, were also detected in our group. There are differences in reports regarding the incidence of small bowel tumors. In the United States, the most common small bowel tumors registered with the National Cancer Data Base are neuroendocrine (carcinoid) (44%), adenocarcinoma (33%) and lymphoma (17%)[13]. In contrast, in a Japanese multicenter study, small bowel tumors were identified in 144 of 1035 subjects (13.9%) who underwent DBE, of which lymphoma and GIST were the most frequent[14]. The hypothesis that adenocarcinoma is the most common tumor was corroborated by Safatle-Ribeiro *et al*[15]. These differences may be ascribed to racial differences and geographical distribution.

To date, approximately 40 different histological types of small bowel tumors, of which approximately two-thirds are malignant, have been identified[16]. Of the 78 small bowel tumors of our study, the detection rate for malignancy was 74.4%. The majority of the lesions were located in the jejunum, followed by the duodenum and ileum, which is similar to the distribution reported in the literature[17]. Most of the small bowel tumors that have been reported occurred primarily in the proximal small bowel (duodenum and jejunum), except for lymphomas, sarcomas and carcinoids[18,19]. In our study, 95.7% of the adenocarcinoma (22/23) cases were found in the proximal small bowel, and only one case was located in the ileum. Among the GIST cases, 78.9% (15/19) were detected in the proximal small bowel. In the 12 patients with lymphomas, the incidence in the jejunum was similar to that of the ileum. All of the lipomas were in the jejunum. Therefore, for patients with no clinical evidence indicating the tumor location, DBE *via* the oral approach is recommended in patients suspected of having tumors, especially adenocarcinoma, GIST and lipoma.

In large-sample studies, OGIB is the leading indication for DBE, and the diagnostic yield for OGIB is 43%-75%[20]. In our 400 patients, OGIB (37.3%, 149/400) was the main reason for DBE, which agreed with previously reported results, followed by abdominal pain (30.8%, 123/400) and chronic diarrhea (10%, 40/400). For small bowel tumors, early symptoms are often absent or nonspecific. The study by Talamonti MS indicated that obstruction, anemia and obscure bleeding were the most common symptoms of primary lesions[21]. In our group of 78 patients with small bowel tumors, the symptoms were not obviously different from those of the other patients; the top three causes for DBE were OGIB, abdominal pain and intestinal obstruction. Therefore, our research indicates that OGIB and abdominal pain were the most common reasons for DBE in both patients with small bowel tumors and patients with other diagnoses.

DBE and CE have diagnostic superiority over other routine procedures, such as push enteroscopy, abdominal CT and small bowel angiography, in detecting small bowel lesions[22]. In small bowel tumor patients, our study demonstrated that DBE had a higher detection rate than CT (85.90% *vs* 72.90%), whereas there was no difference between DBE and CE. Abdominal CT plays a pivotal role in the diagnosis, localization and staging of neoplasms and monitoring the treatment response[23]. At the same time, this examination is convenient and can determine the route of insertion for complementary DBE; therefore, it has become the initial screening method for tumors. However, it is not sufficient for the diagnosis of mucosal or small lesions of the small bowel. In the study of Cheung *et al*[24], tumors measuring less than 10 mm were missed with radiological techniques. CE examination has rapidly gained acceptance as the standard for small bowel evaluation. However, false-positive or false-negative results caused by the unique anatomical features of the small bowel are limitations of capsule endoscopy[25]. Imaoka *et al*[26] reported that two-thirds of patients in whom small bowel tumors were identified had stenosis or ulceration; CE is an inappropriate modality for those who have stenosis. The capsule retention incidence ranges from 9.7%-25% in patients with small bowel tumors, which is higher than the retention incidence in all patients receiving CE and even higher than in patients with small bowel Crohn's disease[27]. DBE examination has no risk of obstruction and allows for the biopsy of tumors, which has high diagnostic value, especially for adenocarcinoma and lymphoma. In our group of 78 tumor patients, 53 cases who underwent an operation were compared regarding the DBE results, and the results indicated that the accuracy rate of DBE in locating small bowel tumors, such as adenocarcinoma, GIST and lymphoma, was very

high. The positive biopsy rates for adenocarcinomas and lymphomas were 71.4% and 60%, respectively. All of the above results indicate that DBE possesses a high value in the qualitative and localization diagnosis of small bowel tumors and provides marked reference values for surgery.

In summary, our study results indicate that DBE examination has high clinical practice value in the diagnosis of tumors and confirms it as a useful diagnostic and therapeutic tool for the investigation of small bowel diseases. DBE can obtain direct visualization and histological characterization of small bowel tumors. DBE should be considered the gold standard for the diagnosis of small bowel tumors because of its unique advantages compared with other procedures[28].

COMMENTS

Background

Small bowel tumors are relatively rare, and the diagnosis of such tumors before surgery was difficult until the advent of double-balloon enteroscopy (DBE) and capsule endoscopy (CE).Compared with CE and other routine examinations used to identify small bowel tumors, DBE has particular advantages because of its diagnostic and therapeutic capabilities.

Research frontiers

Studies are being performed to evaluate the diagnostic value of DBE in small bowel tumors.

Innovations and breakthroughs

This study was a single-center experience in China with a large sample size involving 440 consecutive DBE examinations. The difference between abdominal computed tomography, CE and DBE in the positive rates of detecting small bowel tumors was evaluated. At the same time, the detection rates of various tumors, the location of the lesions, the histological analysis, the reasons for DBE, the accuracy rates for localization and the positive biopsy rates for DBE were also analyzed in detail.

Applications

This study may encourage the use of DBE in the investigation of small bowel tumors. DBE has high diagnostic and therapeutic capabilities in clinical practice; therefore, should be considered as the gold standard for small bowel tumors. In the future, more therapies for small bowel tumors will be finished through DBE.

Terminology

Double-balloon enteroscopy (DBE): a method of enteroscopy that can lead to the observation of the small intestine *via* the mouth or anus with the help of two balloons. One balloon is attached to the tip of the endoscope and the other balloon is attached to the distal end of a soft overtube.

Peer review

This is an interesting paper with important results, which demonstrates the importance of DBE in the diagnosis of small bowel tumors and analyzes the clinical characteristics of 78 tumor patients who underwent DBE.

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BRIEF ARTICLE

Case-matched comparison of laparoscopy-assisted and open distal gastrectomy for gastric cancer

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Abstract

AIM: To compare short- and long-term outcomes of laparoscopy-assisted and open distal gastrectomy for gastric cancer.

METHODS: A retrospective study was performed by comparing the outcomes of 54 patients who underwent laparoscopy-assisted distal gastrectomy (LADG) with those of 54 patients who underwent open distal gastrectomy (ODG) between October 2004 and October 2007. The patients' demographic data (age and gender), date of surgery, extent of lymphadenectomy, and differentiation and tumor-node-metastasis stage of the tumor were examined. The operative time, intraoperative blood loss, postoperative recovery, complications, pathological findings, and follow-up data were compared between the two groups.

RESULTS: The mean operative time was significantly longer in the LADG group than in the ODG group (259.3 ± 46.2 min *vs* 199.8 ± 40.85 min; $P < 0.05$), whereas intraoperative blood loss and postoperative complications were significantly lower (160.2 ± 85.9 mL *vs* 257.8 ± 151.0 mL; 13.0% *vs* 24.1%, respectively, $P < 0.05$). In addition, the time to first flatus, time to initiate oral intake, and postoperative hospital stay were significantly shorter in the LADG group than in the ODG group (3.9 ± 1.4 d *vs* 4.4 ± 1.5 d; 4.6 ± 1.2 d *vs* 5.6 ± 2.1 d; and 9.5 ± 2.7 d *vs* 11.1 ± 4.1 d, respectively; $P < 0.05$). There was no significant difference between the LADG group and ODG group with regard to the number of harvested lymph nodes. The median follow-up was 60 mo (range, 5-97 mo). The 1-, 3-, and 5-year disease-free survival rates were 94.3%, 90.2%, and 76.7%, respectively, in the LADG group and 89.5%, 84.7%, and 82.3%, respectively, in the ODG group. The 1-, 3-, and 5-year overall survival rates were 98.0%, 91.9%, and 81.1%, respectively, in the LADG group and 91.5%, 86.9%, and 82.1%, respectively, in the ODG group. There was no significant difference between the two groups with regard to the survival rate.

CONCLUSION: LADG is suitable and minimally invasive for treating distal gastric cancer and can achieve similar long-term results to ODG.

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Key words: Stomach neoplasms; Gastrectomy; Laparoscopy; Survival; Case matched study

Core tip: We retrospectively analyzed patients treated with laparoscopy-assisted distal gastrectomy (LADG) and compared the surgical and long-term outcomes of LADG and open distal gastrectomy for gastric cancer. Our analysis showed that LADG has the advantages of minimally invasive surgery, rapid recovery, and fewer complications. The effect of lymph node dissection and distance of excision margin were as good as those of

open gastrectomy. Long-term follow-up showed no obvious differences compared to open surgery. LADG can achieve a radical effect similar to that of open surgery.

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INTRODUCTION

Since Kitano *et al*[1] described the first early gastric cancer treated by laparoscopy-assisted distal gastrectomy (LADG), this procedure has rapidly gained popularity in both international and domestic hospitals[2]. Although the minimally invasive effect of LADG is excellent, the therapeutic effects in adenocarcinoma still lack support from long-term follow-up studies. In this study, we performed a 1:1 case matched study to retrospectively analyze patients treated with LADG in our hospital and compared the surgical and long-term outcomes of LADG and open distal gastrectomy (ODG) for gastric cancer.

MATERIALS AND METHODS

Patients

A total of 108 (1:1 matched) consecutive patients who underwent LADG (54) or ODG (54) between October 2004 and October 2007 at the General Surgery Department of Sir Run Shaw Hospital were included in this study. The patients were matched for the following parameters: gender, age (± 5 years), American Society of Anesthesiologists Physical Status score (ASA), time of operation (± 6 mo), extent of lymph node resection (standard D_2), and differentiation and tumor-node-metastasis (TNM) stage of tumor. Clinical and pathological staging was determined according to the American Joint Committee on Cancer (sixth edition) TNM classification scheme.

Surgical technique for gastrectomy

The laparoscope was introduced through a 10-mm infraumbilical trocar while the patient lay in the supine position, followed by placement of two 5-mm assistant ports in the bilateral anterior axillary line in the lower costal margin. The working port was placed to the right external rectus, 2 cm above the umbilicus through a 12-mm trocar, and another 5-mm assistant port was placed in the left corresponding position. These 5 operating trocars were placed in a V-shaped distribution. The surgeon and the second assistant (camera operator) stood on the patient's right and the first assistant stood on the patient's left. The procedure began with the division of the greater omentum and continued with the exposure of the right gastro-omental artery and vein along the gastroduodenal artery and intermediate vein, dissecting at their roots and the infrapyloric lymph nodes. The root of the right gastric artery was exposed along the plane of the common and proper hepatic artery and the duodenohepatic ligament and suprapyloric lymph nodes were dissected. The duodenum was dissected and transected using the endoscopic gastrointestinal anastomosis (endo-GIA) stapler after thorough dissociation of the duodenal ampulla. After retracting the stomach specimen on the left, the lymph nodes along the proximal common hepatic artery, celiac axis, and the root of the splenic artery were dissected in the order described. The root of the left gastric artery was exposed and clipped. We then opened the right diaphragmatic crura and dissected the right cardiac nodes. The stomach specimen was extracted through a 6-cm vertical median incision at the epigastrium and a Billroth II gastrojejunostomy was performed after resection of the stomach specimen.

Variables

The patients' surgical characteristics (operative time, extent of intraoperative hemorrhage, and amount of blood transfused), postoperative recovery (time to first flatus, time to initiate oral intake, complications, and length of postoperative hospital stay), and histopathologic indices (number of resected lymph nodes, surgical margins distance) were observed and compared between the two groups. Follow-up was conducted through an outpatient service, telephone call, or mail in order to determine whether recurrences, metastasis, or death occurred.

Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS®) version 16.0 (SPSS, Inc. Chicago, IL, United States). The differences in the measurement data were compared using the Student's *t* test, and comparisons between groups were tested using the χ^2 test or the Fisher exact probability test. The survival data were estimated using the Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

RESULTS

Preoperative characteristics and postoperative pathologic features

The preoperative characteristics and postoperative pathologic features of the LADG group and the ODG group are summarized in Table 1. There were no differences with respect to preoperative characteristics and postoperative pathologic features between the two groups.

Comparison of surgical indices

Both surgical approaches were completed successfully with no conversion from LADG to open operation. Surgical indices are shown in Table 2. The operative time in the LADG group was longer than that in the ODG group ($P < 0.01$), whereas blood loss and blood infusion frequency were significantly lower ($P < 0.01$). There were no significant differences in the mean number of

Table 1 Perioperative characteristics of patients undergoing gastrectomy

Characteristics	LADG (n = 54)	ODG (n = 54)	P value
Age (yr)	58.2 ± 11.9	58.4 ± 11.6	0.93
Gender (M/F)	40/14	40/14	1.00
BMI index (kg/m^2)	22.6 ± 3.2	21.6 ± 3.9	0.15
Complication (Yes/No)	16/38	18/36	0.42
ASA (Ⅰ/Ⅱ)	24/30	24/30	1.00
Tumor size (cm)	3.7 ± 1.9	3.5 ± 1.5	0.17
Histology (well/moderate/low/signet ring)	19/10/14/11	19/10/14/11	1.00
Lymph node metastasis (+/-)	27/27	25/29	0.85
TNM stage (ⅠA/ⅠB/Ⅱ/ⅢA/ⅢB)	22/8/9/10/15	22/8/9/10/15	1.00

ODG: Open distal gastrectomy; LADG: Laparoscopy-assisted distal gastrectomy; TNM: Tumor-node-metastasis; ASA: American Society of Anesthesiologists Physical Status score; M: Male; F: Female.

Table 2 Comparison

Variables	LADG (n = 54)	ODG (n = 54)	P value
Surgical indices			
Operative time (min)	259.3 ± 46.2	199.8 ± 40.8	< 0.01
Blood loss (mL)	160.2 ± 85.9	257.8 ± 151.0	< 0.01
Intraoperative blood infusion (yes/no)	1/53	13/41	< 0.01
Number of retrieved lymph nodes	27.9 ± 7.8	27.7 ± 10.1	0.94
Distance of the proximal margin (cm)	3.6 ± 1.9	4.4 ± 2.1	0.27
Distance of the distal margin (cm)	4.3 ± 2.1	4.8 ± 2.3	0.30
Postoperative recovery			
First flatus (d)	3.9 ± 1.4	4.4 ± 1.5	0.03
Initiate fluid intake (d)	4.6 ± 1.2	5.6 ± 2.1	< 0.01
Initiate semifluid intake (d)	6.0 ± 1.7	7.4 ± 2.4	< 0.01
Hospital stay (d)	9.5 ± 2.7	11.1 ± 4.1	0.02

ODG: Open distal gastrectomy; LADG: Laparoscopy-assisted distal gastrectomy.

retrieved lymph nodes between the two groups.

Comparison of postoperative recovery

Postoperative recovery in the two groups is shown in Table 2. The time to first flatus, time to initiate oral intake, and postoperative hospital stay were significantly shorter in the LADG group than in the ODG group ($P < 0.05$).

Comparison of postoperative complications and mortality rate

Seven (13%) patients experienced postoperative complications after LADG. One patient developed short-term gastric emptying disorder and was discharged on the 16th postoperative day after treatment with gastrointestinal decompression, gastrointestinal prokinetic agents (GIPA), and total parenteral nutrition (TPN) support therapy. One patient developed chylous fistula and was discharged 14 d after surgery following treatment with short-term fasting and TPN support. Two patients developed pulmonary infections and both patients recovered after antibiotic treatment. Three patients developed abdominal cavity effusions with complicating inflammation, one was reversed after conservative therapy, the other two were treated with abscess needle puncture and drainage under computed tomographic (CT) guidance and were discharged 16, 22, and 24 d after surgery, respectively.

Sixteen (24.1%) patients in the ODG group developed postoperative complications. One patient developed an anastomotic leak and was discharged on the 36th postoperative day after undergoing re-operation. In two patients with intra-abdominal bleeding, one was reversed after conservative therapy, the other underwent re-operation, and they were discharged 12 and 13 d after surgery, respectively. Of four patients with abdominal cavity effusions with complicating infection, one underwent laparotomy, abdominal cavity flushing drainage and was discharged 40 d after surgery, and the others were treated with abscess needle puncture and drainage under CT guidance and were discharged 18, 20, and 21 d after surgery, respectively. Three patients had pulmonary infections and all recovered after antibiotic treatment. Two patients had short-term gastric emptying disorder and were discharged on the 20th and 26th postoperative day, respectively, after treatment with gastrointestinal decompression, GIPA and TPN support. Two patients developed chylous fistula and were discharged 15 and 16 d after surgery, respectively, following treatment with short-term fasting and TPN support. Two patients had wound infections which were reversed after the incision was opened and dressed. No preoperative deaths occurred in either group. Comparisons of postoperative complications and mortality rates are summarized in Table 3.

Comparison of long-term results

Of the 108 identified patients, 96 (88.9%) were followed up and 12 were lost to follow-up. Follow-up data were available for 49 (90.1%) and 47 (87.0%) of patients treated with LADG and ODG, respectively. The mean follow-up was 60 mo (range, 5-97 mo).

In the LADG group, 11 (20.4%) patients experienced recurrence and 8 died due to the disease: one stage IA patient developed recurrence and he is still alive 64 mo after operation. Another stage I B patient died 51 mo after operation. Two stage Ⅱ patients had recurrence, one is alive 57 mo after operation, and the other died. Five stage ⅢA patients experienced recurrence, four of whom died during the follow-up period, and the remaining patient is alive 56 mo after operation. Two stage ⅢB patients died of the disease 14 and 59 mo after operation, respectively.

In the ODG group, nine (16.7%) patients had recurrence and 8 patients died of metastatic disease: three stage IB patients died due to the disease 9, 11, 49 mo after operation, respectively; two stage Ⅱ patients experienced recurrence, one of whom died, and the other is alive 79 mo after operation; another four stage ⅢA patients also had recurrence, and they died 6, 11, 15, and 31 mo after operation, respectively.

The 1-, 3-, and 5-year disease-free survival rates were

Table 3 Comparison of postoperative complications and mortality rate *n* (%)

Variables	LADG	ODG	*P* value
Overall	7 (13.0)	16 (24.1)	0.03
Anastomotic leakage	0	1	
Anastomotic stenosis	0	0	
Intra-abdominal bleeding	0	2	
Abdominal cavity effusion complicating infection	3	4	
Pulmonary infection	2	3	
Gastric emptying disorder	1	2	
Chylous fistula	1	2	
Wound infection	0	2	
Reoperation	0 (0.0)	3 (5.6)	0.24
Mortality	0 (0.0)	0 (0.0)	1.00

ODG: Open distal gastrectomy; LADG: Laparoscopy-assisted distal gastrectomy.

94.3%, 90.2%, and 76.7%, respectively, in the LADG group and 89.5%, 84.7%, and 82.3%, respectively, in the ODG group. The 1-, 3-, and 5-year overall survival rates were 98.0%, 91.9%, and 81.1%, respectively, in the LADG group and 91.5%, 86.9%, and 82.1%, respectively, in the ODG group. There were no significant differences in these values between the two groups ($P > 0.05$) (Figure 1).

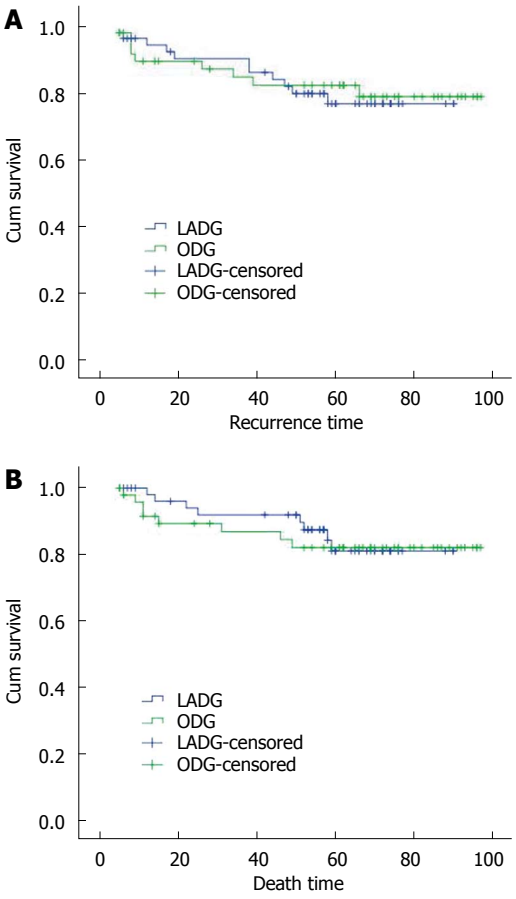

Figure 1 Curves showing disease-free survival (A) and overall survival (B) after gastrectomy. ODG: Open distal gastrectomy; LADG: Laparoscopy-assisted distal gastrectomy.

DISCUSSION

Since the first successful LADG by Kitano *et al*[1] for early gastric cancer in 1994, the use of LADG has significantly increased in both international and domestic hospitals. More and more studies, including some randomized controlled trials, have demonstrated the advantages of LADG in the treatment of early gastric cancer such as earlier recovery of ambulation and bowel movement, less pain, and a lower rate of complications. These studies confirmed that LADG is a safe and feasible surgical method[3-5].

Kitano *et al*[6] reported the clinical findings of a multicenter trial which included 1491 cases of laparoscopy-assisted distal gastrectomy at 18 surgery centers, and found a complication rate of 12%, death rate of 0%, and recurrence rate of 0.4%. Prospective studies by Dulucq *et al*[7] revealed that postoperative complications and length of hospital stay in the LADG group were reduced compared with those in the ODG group. In the present study, complications, time to first flatus, time to initiate oral intake, and postoperative hospital stay were all significantly shorter in the LADG group than in the ODG group ($P < 0.05$), showing the advantages of LADG during postoperative recovery. Several publications have reported that the rate of surgical complications following LAG are in the range of 3.8%-26.7%[8-11], this rate was 13% in our study and was significantly lower than that in the ODG group. There was no preoperative death in our study; however, a recent survey showed that the mortality rate for open gastric cancer radical gastrectomy was 4%-6%[12].

Some studies have described the disadvantages of LADG compared to ODG, which include increased operative time similar to that in our study[13-16]. Operator experience, familiarity with instruments, and degree of assistant compliance are major factors influencing the operative time. During initial LADG procedures in our center, the operative time was often more than 300 min due to the above-mentioned reasons; these factors may be responsible for the significantly longer average operative time in the LADG group than in the ODG group. Other reports have also indicated that operative time with experienced surgeons was similar for LADG and ODG[17,18].

Similar to most reports, the intraoperative blood loss in the LADG group was less than that in the ODG group[5,19], as was blood transfusion, which reiterated the importance of careful laparoscopic manipulation during LADG. In addition, magnified vision also creates conditions for careful laparoscopic manipulation. Lack of blood is a common problem faced by many hospitals, especially in developing countries such as China; therefore, less invasive laparoscopic surgery can reduce the clinical requirement for blood and lower the rate of complications associated with blood transfusion such as virus infection and allergic reaction.

Radical resection of the tumor lymphatic drainage area should include complete resection and well incised

margins. Laparoscopic gastric cancer D_2 lymph node dissection is difficult and requires high technical expertise. Thus, whether laparoscopic techniques can be applied in the treatment of advanced gastric cancer is still controversial. However, the number of laparoscopically dissected lymph nodes is closely related to the surgical technique, and some studies have shown that there was no difference in the number of retrieved lymph nodes between LAG and OG[20,21]. Our analysis showed similar results in that D_2 lymph node dissection can be completed during laparoscopy. Park *et al*[22] evaluated the long-term results of 239 patients who underwent LADG for the treatment of advanced gastric cancer. They found that the major recurrence was distant metastasis, whereas lymph node relapses were most frequent in para-aortic or distant lymph node metastasis; therefore, we believe that the dissection of lymph nodes around the stomach can be performed efficiently by LADG. In our study, the mean distance of the proximal and the distal margin in the LADG group was 3.6 ± 1.9 cm and 4.3 ± 2.1 cm, respectively, without residual cancer cells and no statistical difference compared with the OG group. We conclude that laparoscope-assisted radical gastrectomy may help achieve results similar to those of abdominal opening on margin distance. Port-site metastasis caused by intraoperative pneumoperitoneum of LADG is another controversial issue. Port-site metastases were not reported in our study, similar to other studies, thus we conclude that pneumoperitoneum does not contribute to a higher risk of port-site metastasis.

Whether LADG can achieve effective radical cancer excision similar to that by ODG needs to be confirmed through long-term outcomes, however, large-volume long-term studies are still lacking. In a prospective trial conducted by Huscher *et al*[11], the long-term results of LAG and OG were similar, but the scope of lymph node dissection was identical. A retrospective analysis conducted by Hamabe *et al*[23] revealed that the long-term results did not differ significantly between LAG and OG; however, because the application of LAG increased gradually, whereas the application of OG decreased gradually over the entire study period, the former had a short follow-up time. However, during the 10-year study, improvements in the chemotherapy program will certainly have an impact on the study results. Sato *et al*[24] analyzed the difference between OG and LAG in relation to D1, D1+, or D2 lymph node dissection using a hierarchical approach and found that the long-term results of LAG were comparable to those of OG; however, the pathological stage in the LAG group was significantly earlier than that in the OG group ($P < 0.001$), which seriously affected the credibility of the analysis. Pak *et al*[25] analyzed the follow-up of 714 patients who underwent LAG and calculated the 5-year survival rate of different TNM stages, however, their findings were not compared with those of open surgery over the same period. In addition, their follow-up duration was not long enough, as the median follow-up was less than 50 mo. Although postoperative recurrence after radical gastrectomy for cancer usually occurs within 36 mo, about 12% of cases with recurrence occur after 36 mo[26,27]. Therefore, prolonged follow-up can result in more reliable survival analysis data.

In our study, the follow-up duration was long enough as the median follow-up duration was 60 mo. In addition, we found no difference in the disease-free survival or overall survival rate between the two groups, after conducting a matched-pair design to eliminate the effects of confounding factors, including gender, age, operative period, ASA status, the extent of lymph node resection, and differentiation and TNM stage of the tumor. These results suggest that the LADG is as good as ODG with regard to long-term outcomes. The matched-pair design makes the results more credible. However, this study has several limitations, including its relatively small sample size, no survival analysis after stratification according to the TNM stage, and the use of matching research, which is a type of retrospective analysis that cannot replace prospective trials.

In conclusion, our analysis showed that LADG has the advantages of minimally invasive surgery, rapid recovery, and fewer complications. The effect of lymph node dissection and distance of excision margin were as good as those of open gastrectomy. Long-term follow-up of LADG patients showed no obvious differences compared to open surgery. We believe that LADG can achieve a radical effect similar to that of open surgery in patients with gastric cancer.

COMMENTS

Background

More and more studies, including some randomized controlled trials, have demonstrated the advantages of laparoscopy-assisted distal gastrectomy (LADG) in the treatment of early gastric cancer such as earlier recovery of ambulation and bowel movement, less pain, and a lower rate of complications. These studies have confirmed that LADG is a safe and feasible surgical method, but the therapeutic effects in adenocarcinoma still lack support from long-term follow-up studies.

Research frontiers

Although the minimally invasive effect of LADG is excellent, the therapeutic effects in adenocarcinoma still lack support from long-term follow-up studies. Whether LADG can achieve effective radical cancer excision similar to that by open distal gastrectomy (ODG) requires to be confirmed through long-term outcomes, however, large-size long-term studies are still lacking.

Innovations and breakthroughs

In this study, the authors performed a 1:1 case matched study to retrospectively analyze the patients treated by LADG and compared the surgical and long-term outcomes of LADG and ODG for gastric cancer. This study showed that LADG is suitable and minimally invasive for treating distal gastric cancer and can achieve similar long-term results to ODG.

Applications

This study showed that LADG has the advantages of minimally invasive surgery, rapid recovery, and fewer complications. The effects of lymph node dissection and distance of the excision margin are as good as those of open gastrectomy. Long-term follow-up showed no obvious differences compared to open surgery. LADG can achieve a radical effect similar to that of open surgery in patients with gastric cancer. These findings are helpful in decision-making for the treatment of resectable gastric cancer.

Peer review

This is a nice paper, which is worth reading and publication. The subject and findings are significant in clinical practice, especially in the field of laparoscopy-assisted surgery. Overall, the manuscript is well prepared.

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META-ANALYSIS

Diagnostic accuracy of endoscopic ultrasound in pancreatic neuroendocrine tumors: A systematic review and meta analysis

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Abstract

AIM: To detect pancreatic neuroendocrine tumors (PNETs) has been varied. This study is undertaken to evaluate the accuracy of endoscopic ultrasound (EUS) in detecting PNETs.

METHODS: Only EUS studies confirmed by surgery or appropriate follow-up were selected. Articles were searched in Medline, Ovid journals, Medline nonindexed citations, and Cochrane Central Register of Controlled Trials and Database of Systematic Reviews. Pooling was conducted by both fixed and random effects model).

RESULTS: Initial search identified 2610 reference articles, of these 140 relevant articles were selected and reviewed. Data was extracted from 13 studies (n = 456) which met the inclusion criteria. Pooled sensitivity of EUS in detecting a PNETs was 87.2% (95%CI: 82.2-91.2). EUS had a pooled specificity of 98.0% (95%CI: 94.3-99.6). The positive likelihood ratio of EUS was 11.1 (95%CI: 5.34-22.8) and negative likelihood ratio was 0.17 (95%CI: 0.13-0.24). The diagnostic odds ratio, the odds of having anatomic PNETs in positive as compared to negative EUS studies was 94.7 (95%CI: 37.9-236.1). Begg-Mazumdar bias indicator for publication bias gave a Kendall's tau value of 0.31 (P = 0.16), indication no publication bias. The P for χ^2 heterogeneity for all the pooled accuracy estimates was > 0.10.

CONCLUSION: EUS has excellent sensitivity and specificity to detect PNETs. EUS should be strongly considered for evaluation of PNETs.

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Key words: Endoscopic ultrasound; Ultrasound; Endosonography; Pancreatic mass; Neuroendocrine tumors; Sensitivity; Specificity; Positive predictive value; Negative predictive value

Core tip: The published data on the diagnostic accuracy of endoscopic ultrasound (EUS) for detection of pancreatic neuroendocrine tumors is varied. We conducted a comprehensive review of the published literature to as-

sess the diagnostic accuracy of EUS in this setting. Our systematic review and meta-analysis has demonstrated an excellent sensitivity and specificity of EUS in this setting compared to previously published literature of other imaging modalities such as transabdominal ultrasound, computed tomography, and magnetic resonance imaging.

Puli SR, Kalva N, Bechtold ML, Pamulaparthy SR, Cashman MD, Estes NC, Pearl RH, Volmar FH, Dillon S, Shekleton MF, Forcione D. Diagnostic accuracy of endoscopic ultrasound in pancreatic neuroendocrine tumors: A systematic review and meta analysis. *World J Gastroenterol* 2013; 19(23): 3678-3684 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3678.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3678

INTRODUCTION

Neuroendocrine tumors of the gastrointestinal tract are rare, accounting for less than 1% of all malignancies with an estimated annual incidence of 1-4 per 100000; however, they may lead to significant morbidity and mortality[1,2]. These tumors are difficult to diagnosis, treat, and have a propensity for metastasis prior to their diagnosis given that many to not become clinically apparent until late in their course. These tumors may be found throughout the gastrointestinal tract; however, the pancreas is an area where neuroendocrine tumors are commonly discovered[3,4].

Neuroendocrine tumors of the pancreas (PNETs) may be functional or non-functional and are mostly sporadic, although some are associated with other genetic diseases[1]. Functional PNETs often secrete active substances, such as insulin, somatostatin, gastrin, glucagon, or vasoactive intestinal peptide, which may allow them to be discovered earlier[1,5]. However, some of these PNETs are non-functional, secreting non-active substances, such as chromogranin A[1]. Serological tests have been used to determine levels of these compounds, leading to an enhanced ability to diagnosis PNET. However, these tumors tend to have metastasized by the time they are diagnosed, especially in non-functioning PNETs. Many imaging modalities have been utilized for PNETs, including trans-abdominal ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI) with significant limitations. These imaging techniques are able to detect PNETs in 9%-48% with an estimated sensitivity of 29%-60%[6-8]. Given the need for improved imaging techniques, endoscopic ultrasound (EUS) has been evaluated as a possible diagnostic tool for PNETs.

Since its early introduction in the early 1990's, EUS has emerged as a safe and accurate technique for the diagnosis, stage, and treat a variety of lesions. A particularly useful aspect of EUS is the enhanced imaging of the pancreas. There are currently several reports of EUS in correctly detecting PNETs. However, the accuracy of these results varies across centers. To the best of our knowledge, a meta-analysis summarizing these results has not been performed. The purpose of this investigation is to review the world literature regarding the accuracy of EUS in detecting PNET.

MATERIALS AND METHODS

Study selection criteria

Studies evaluating the use of EUS to characterize pancreatic neuroendocrine tumors with a gold standard (either confirmed by surgery or appropriate follow-up) were selected. From this pool, only studies from which a 2 × 2 table could be constructed for true positive, false negative, false positive and true negative values were included.

Data collection and extraction

Articles were searched in MEDLINE (through PubMed, an electronic search engine for published articles and Ovid), PubMed, Ovid Journals, EMBASE, Cumulative Index for Nursing and Allied Health Literature, ACP Journal Club, DARE, International Pharmaceutical Abstracts, old Medline, Medline non-indexed citations, OVID Healthstar, and Cochrane Central Register of Controlled Trials and Database of Systematic Reviews (Central). The search was performed from January 1966 to January 2012. The terms used for search were endoscopic ultrasound, EUS, ultrasound, endosonography, pancreatic mass, neuroendocrine tumors, sensitivity, specificity, positive predictive value, and negative predictive value. Study authors were contacted when the required data could not be determined from the publications. Two by two tables were constructed with the data extracted from each study. Two authors (Puli SR, Bechtold ML) independently searched and extracted the data using an abstraction form. Any differences were resolved by mutual agreement. The agreement between reviewers for the collected data was quantified using the Cohen's κ[9].

Quality of studies

Clinical trials designed with control and treatment arms can be assessed for quality of the study. A number of criteria have been used to assess the quality of a study (*e.g.*, randomization, selection bias of the arms in the study, concealment of allocation, and blinding of outcome)[10,11]. There is no consensus on how to assess studies designed without a control arm. Hence, these criteria do not apply to studies without a control arm[11]. Therefore, for this meta-analysis and systematic review, studies were selected based on completeness of the data and inclusion criteria. Completeness was defined as data available for true positive, false negative, false positive and true negative values of the diagnostic test (EUS). Quality Assessment of Studies of Diagnostic Accuracy Included in Systematic Reviews (QUADAS) criteria has been proposed to evaluate quality of diagnostic studies[12,13]. This was used to evaluate the studies on 14 items described in the QUADAS criteria.

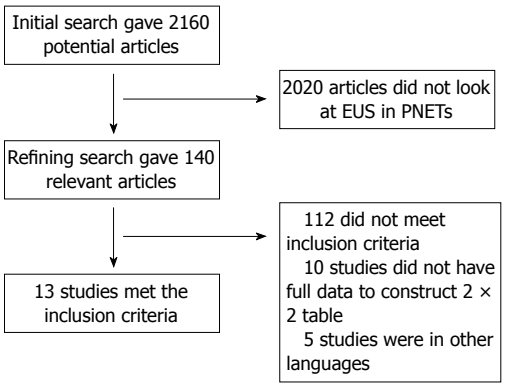

Figure 1 Search results. EUS: Endoscopic ultrasound; PNETs: Pancreatic neuroendocrine tumors.

Statistical analysis

Meta-analysis for the accuracy of EUS in diagnosing PNETs was performed by calculating pooled estimates of sensitivity, specificity, likelihood ratios, and diagnostic odds ratios. Pooling was conducted using both Mantel-Haenszel Method (fixed effects model) and DerSimonian Laird Method (random effects model). The confidence intervals were calculated using the F distribution method[14]. Forrest plots were drawn to examine how the point estimates in each study related to the summary pooled estimate. For 0 value cells, a 0.5 was added as described by Cox[15]. The heterogeneity of the sensitivities and specificities were tested by applying the likelihood ratio test[16]. The heterogeneity of likelihood ratios and diagnostic odds ratios were tested using Cochran's Q test based upon inverse variance weights[17]. Heterogeneity among studies was also tested by using summary receiver operating characteristic (SROC) curves. SROC curves were used to calculate the area under the curve (AUC). The affect of publication and selection bias on the summary estimates was tested by Egger bias indicator[18]. Also, funnel plots were constructed to evaluate potential publication bias using the standard error and diagnostic odds ratio[19,20].

RESULTS

Initial search identified 2610 reference articles, of these 140 relevant articles were selected and reviewed. Data was extracted from 13 studies[21-33] (n = 456) which met the inclusion criteria. Search results are shown in Figure 1. All the pooled estimates given are estimates calculated by the fixed effects model. The change adjusted agreement analysis between the reviewers for data collected separately gave a kappa value of 1.0. QUADAS criteria to evaluate the quality of studies demonstrated that all the studies fulfilled 4 to 5 out of 14 described criterion.

Accuracy of EUS to detect PNETs

Pooled sensitivity of EUS in detecting PNETs was 87.2% (95%CI: 82.2-91.2). Forrest plot in Figure 2A shows the sensitivity of EUS in individual included studies. EUS had a pooled specificity of 98.0% (95%CI: 94.3-99.6). Figure 2B shows the specificity from various studies. The positive likelihood ratio (+LR) of EUS was 11.1 (95%CI: 5.34-22.8) and negative likelihood ratio (-LR) was 0.17 (95%CI: 0.13-0.24). The diagnostic odds ratio (DOR), the odds of having anatomic PNETs in positive as compared to negative EUS studies was 94.7 (95%CI: 37.9-236.1). All the pooled estimates calculated by fixed and random effect models were similar. SROC curves showed an area under the curve of 0.94. Figure 3 shows SROC curve and the area under the curve. Egger bias indicator for publication bias gave a value of 1.39 (95%CI: -1.52-4.32, P = 0.31), indication no publication bias. Funnel plot in Figure 4 also shows that there is no publication bias in the included studies. The P for chi-squared heterogeneity for all the pooled accuracy estimates was > 0.10.

Subgroup analysis was performed to see how EUS performs in detecting an Insulinoma or a Gastrinoma in the pancreas.

Accuracy of EUS to detect an insulinoma in the pancreas

Data was extracted from 9 studies (n = 242) which met the inclusion criteria. Pooled sensitivity of EUS in detecting a Pancreatic Insulinoma was 87.5% (95%CI: 81.2-92.3). EUS had a pooled specificity of 97.4% (95%CI: 90.8-99.7). The +LR of EUS was 8.2 (95%CI: 3.7-18.3) and -LR was 0.17 (95%CI: 0.12-0.26). The DOR, the odds of having anatomic Pancreatic Insulinoma in positive as compared to negative EUS studies was 67.6 (95%CI: 22.7-200.9). All the pooled estimates calculated by fixed and random effect models were similar. SROC curves showed an area under the curve of 0.94. Egger bias indicator for publication bias gave a value of -0.05 (95%CI: -4.13-4.04, P = 0.98), indication no publication bias. The P for chi-squared heterogeneity for all the pooled accuracy estimates was > 0.10.

Accuracy of EUS to detect gastrinoma in the pancreas

Five EUS studies (n = 122) looked at detecting Gastrinomas in the pancreas. Pooled sensitivity of EUS in detecting a gastrinoma in the pancreas was 84.5% (95%CI: 72.6-92.7). EUS had a pooled specificity of 95.3% (95%CI: 86.9-99.0). The positive likelihood ratio of EUS was 10.5 (95%CI: 4.3-25.5) and negative likelihood ratio was 0.25 (95%CI: 0.13-0.47). The diagnostic odds ratio, the odds of having anatomic gastrinoma in positive as compared to negative EUS studies was 57.3 (95%CI: 15.1-217.2). All the pooled estimates calculated by fixed and random effect models were similar. SROC curves showed an area under the curve of 0.94. Egger bias indicator for publication bias gave a value of -0.74 (95%CI: -15.19-13.72, P = 0.88), indication no publication bias. The P for χ^2 heterogeneity for all the pooled accuracy estimates was > 0.10.

DISCUSSION

Localizing or detecting a neuroendocrine neoplasm in the pancreas helps not only with the planning of treatment but also when detected early might improve overall prog-

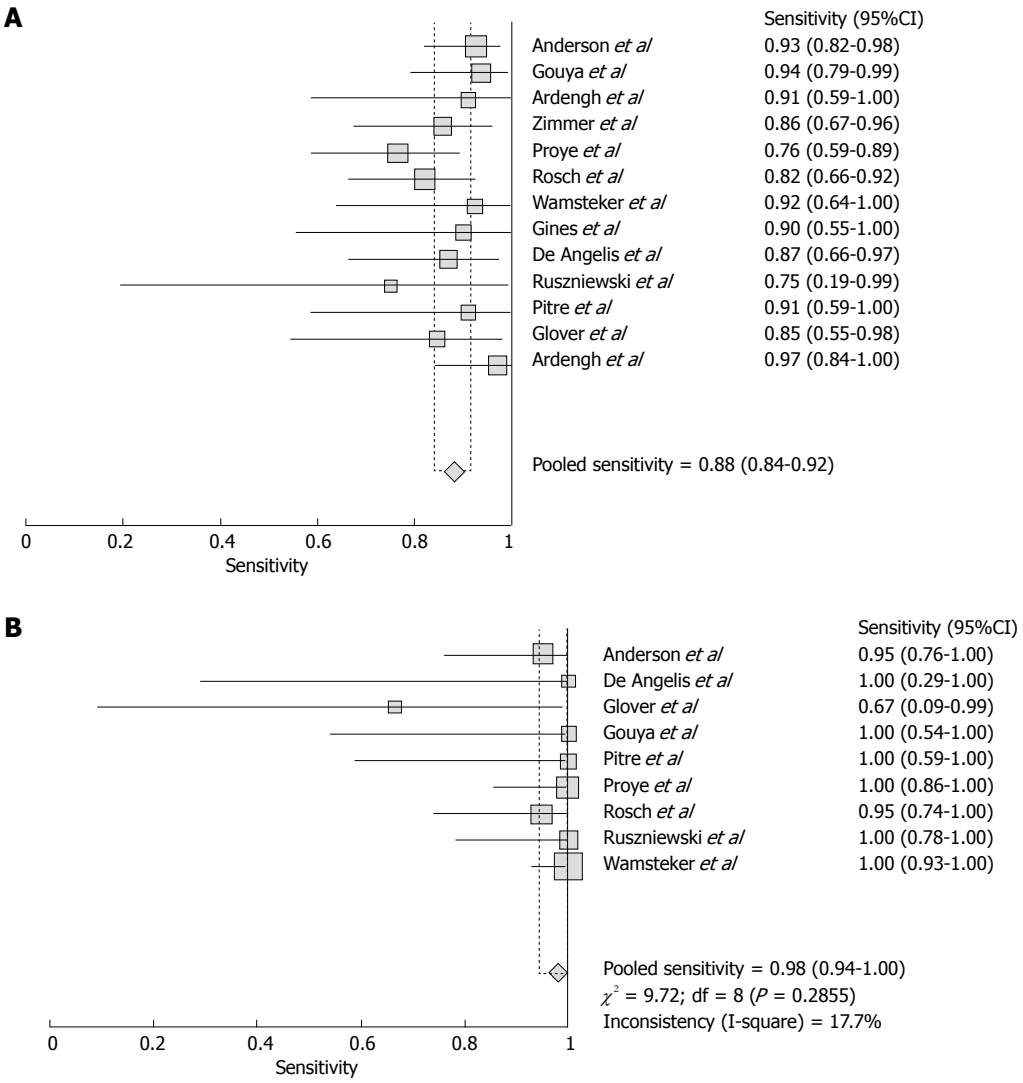

Figure 2 Forrest plot. A: Forrest plot showing sensitivity of endoscopic ultrasound (EUS) to detect pancreatic neuroendocrine tumor; B: Forrest plot showing specificity of EUS to detect pancreatic neuroendocrine tumors.

nosis. Over the past sixteen years since the introduction of EUS, this minimally invasive technique has emerged as the premiere modality to confirm pancreatic neoplasms. In this meta-analysis we sought to pool and compare the findings of 13 high quality studies concerned with the performance of EUS in the evaluation of PNETs.

The strengths of this analysis are that the literature was reviewed and data was extracted independently by two independent reviewers. Comparison of their analyses indicates excellent agreement. This meta-analysis and systematic review was written in accordance with the proposal for reporting by the Quality of Reporting of Meta-analyses statement[34]. A standardized extraction algorithm was applied and only studies which fulfilled at least four of the QUADAS criterion were included. Additionally, extensive efforts were made to ensure that the true positive, false positive, true negative and false negative results for all studies were either gleaned from the literature or acquired via direct communication with the investigators. Egger bias estimates as well as funnel plots were performed and both methods suggest that there was no significant publication bias. Since this manuscript looks at diagnostic accuracy of a test, the study design for this meta-analysis and systematic review followed the guidelines of Standards for Reporting of Diagnostic Accuracy initiative[35].

A core finding of this meta-analysis is that in patients with symptomatic PNETs, EUS had high sensitivity (88%) and specificity (98%) in localizing the lesion to the pancreas. EUS as a diagnostic test has a very high DOR to detect PNETs (about 95 times). If EUS localizes the lesion to the pancreas, the odds of having the correct histological neuroendocrine tumor in the pancreas is 95 times.

Additional performance characteristics for EUS were assessed in this meta-analysis which demonstrate a high +LR and low -LR. The higher the positive likelihood ratio, the better the diagnostic test performs in correctly identifying the true disease state. On the flip side, negative likelihood ratio of a diagnostic test is a measure of how well the test correctly excludes a disease stage. The diagnostic tests ability to exclude a disease state is better

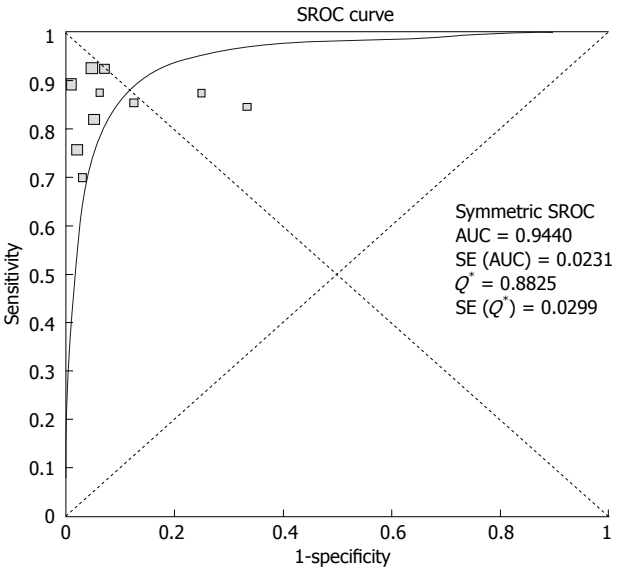

Figure 3 Summary receiver operating characteristic curves of endoscopic ultrasound to detect pancreatic neuroendocrine tumors. AUC: Area under the curve; SROC: Summary receiver operating characteristic.

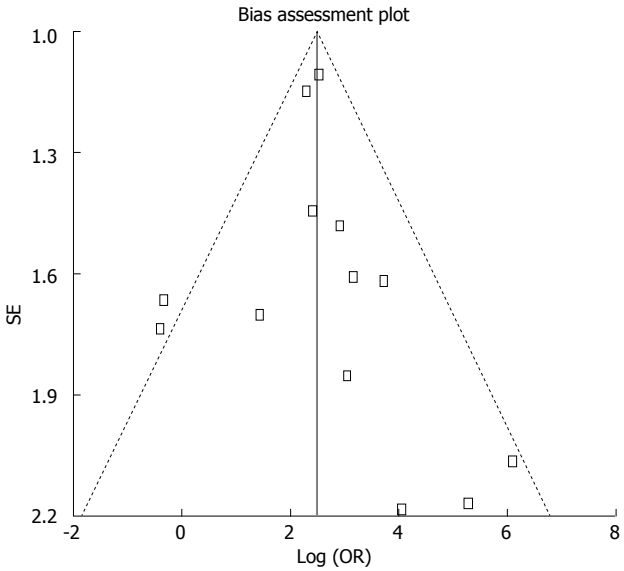

Figure 4 Funnel plot showing no publication bias in the studies included in this meta-analysis.

if the negative likelihood ratio is lower. For PNETs, EUS has a high positive likelihood ratio and a low negative likelihood ratio. This indicates that EUS performs well in excluding as well as correctly localizing neuroendocine tumor within the pancreas.

In a subgroup analysis to look at performance of EUS to correctly diagnose Insulinomas, EUS had high sensitivity (88%) and specificity (97%). Also, EUS had high sensitivity (85%) and specificity (95%) to detect Gastrinomas in pancreas.

A strength of this meta-analysis is that there was no heterogeneity among the studies included in this analysis. Heterogeneity among different studies was evaluated not only with test of heterogeneity but also by drawing SROC curves and finding the AUC. An AUC of 1 for any diagnostic test indicates that the test is excellent. SROC curves for EUS showed that the value of AUC was very close to 1, indicating that EUS is an excellent diagnostic test to detect PNETs.

One limitation of this meta-analysis is that none of the included studies were multicentre or randomized trials. The included studies were small in size indicating the low incidence of neuroendocrine tumors among general population. Studies on EUS with statistical significance tend to be published and cited leading to publication bias. Additionally smaller studies may show larger treatment effects due to fewer case-mix differences (*e.g.*, patients with only early localized *vs* late metastatic disease) than larger studies. This publication and selection bias may affect the summary estimates in any meta-analysis. This bias can be estimated by Egger bias indicators and construction of Funnel plots. In our meta-analysis and systematic review, bias calculations using Harbord-Egger bias indicator[18] showed no statistically significant bias. Furthermore, analysis using Funnel plots showed no significant publication bias among the studies included in this analysis.

At this time the role of EUS to detect PNETs is as an adjunct to the imaging modalities such as CT scans. This is especially true when they are functional and when present in an early localized stage. More recently a single center study undertaken by Khashab *et al*[36] showed the despite improvement in CT technology which has increased detection rates, they still missed PNETs that were smaller in size.

A subgroup analysis to see if FNA could improve the diagnostic accuracy could not be performed as there were only two studies[28,33] that included FNA data for PNETs. In a meta-analysis of 41 studies by Puli *et al*[37], the accuracy of EUS-FNA in the setting of solid pancreatic mass was analysed which showed a pooled sensitivity and specificity of 86.9% (95%CI: 85.5-87.9) and 95.8% (95%CI: 94.6-96.7) respectively. Given these findings it would make sense to probably conclude that EUS-FNA could replicate similar diagnostic characteristics in PNETs. In additional, Khashab *et al*[36] also reported increased diagnostic accuracy of EUS in detected lesions to the pancreas when the were smaller in size. This was especially true for functional PNETs which tend to present early due to active peptides.

In conclusion, EUS has excellent sensitivity and specificity to localize PNETs approaching 100%. In a subgroup analysis, EUS had high sensitivity and specificity to detect Insulinoma or Gastrinoma in the pancreas. Though the studies in literature are small studies, EUS should be strongly considered for evaluation of PNETs.

COMMENTS

Background

Biochemically active neuroendocrine tumors often arise from the pancreas and are preceded by hormone related symptoms before metastasis. They are often small early on and could be missed on traditional imaging such as abdominal ultrasound, computed tomography (CT), and magnetic resonance imaging.

However, the performance characteristics of endoscopic ultrasound (EUS) from previously published studies have demonstrated varying results.

Research frontiers

To our knowledge there is no published meta-analysis that has reported the diagnostic accuracy of EUS in neuroendocrine tumor of pancreas (PNETs). Several small studies have demonstrated varying results. This study is undertaken to assess pooled estimates on the diagnostic accuracy of EUS in early PNETs.

Innovations and breakthroughs

EUS has excellent sensitivity and specificity in detecting PNETs both approaching close to 100%. A subgroup analysis is also performed for pancreatic functional PNETs *i.e.*, gastrinoma and insulinoma which showed high sensitivity and specificity. This gives additional diagnostic option in patients undergoing conventional imaging such as CT scan and scintigraphy with higher diagnostic accuracy compared to previously published data of the former tests.

Applications

EUS can be used to identify pancreatic PNETs with high degree of diagnostic accuracy.

Terminology

EUS has a very high sensitivity and specificity in PNETs especially in early stages aiding in early diagnosis and potential treatment.

Peer review

The current paper of systematic review and meta-analysis investigates the diagnostic accuracy of EUS in diagnosis of PNETs. The statistical analysis performed in this study produced reliable results.

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CASE REPORT

Endoscopic transluminal pancreatic necrosectomy using a self-expanding metal stent and high-flow water-jet system

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Abstract

Walled-off pancreatic necrosis and a pancreatic abscess are the most severe complications of acute pancreatitis. Surgery in such critically ill patients is often associated with significant morbidity and mortality within the first few weeks after the onset of symptoms. Minimal invasive approaches with high success and low mortality rates are therefore of considerable interest. Endoscopic therapy has the potential to offer safe and effective alternative treatment. We report here on 3 consecutive patients with infected walled-off pancreatic necrosis and 1 patient with a pancreatic abscess who underwent direct endoscopic necrosectomy 19-21 d after the onset of acute pancreatitis. The infected pancreatic necrosis or abscess was punctured transluminally with a cystostome and, after balloon dilatation, a non-covered self-expanding biliary metal stent was placed into the necrotic cavity. Following stent deployment, a nasobiliary pigtail catheter was placed into the cavity to ensure continuous irrigation. After 5-7 d, the metal stent was removed endoscopically and the necrotic cavity was entered with a therapeutic gastroscope. Endoscopic debridement was performed *via* the simultaneous application of a high-flow water-jet system; using a flush knife, a Dormia basket, and hot biopsy forceps. The transluminal endotherapy was repeated 2-5 times daily during the next 10 d. Supportive care included parenteral antibiotics and jejunal feeding. All patients improved dramatically and with resolution of their septic conditions; 3 patients were completely cured without any further complications or the need for surgery. One patient died from a complication of prolonged ventilation severe bilateral pneumonia, not related to the endoscopic procedure. No procedure related complications were observed. Transluminal endoscopic necrosectomy with temporary application of a self-expanding metal stent and a high-flow water-jet system shows promise for enhancing the potential of this endoscopic approach in patients with walled-off pancreatic necrosis and/or a pancreatic abscess.

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Key words: Acute necrotizing pancreatitis; Walled off pancreatic necrosis; Endoscopic necrosectomy; Self-expanding metal stent; Water-jet system

Core tip: The endoscopic transluminal management of pancreatic necrosis and/or pancreatic abscess is associated with good initial and long-term clinical success, with acceptable morbidity and mortality rates. The advantages of endoscopic management are related to its minimal invasiveness. The combination of multiple endoscopic approaches is designed to achieve the goals of any treatment strategy.

Hritz I, Fejes R, Székely A, Székely I, Horváth L, Sárkány Á, Altorjay Á, Madácsy L. Endoscopic transluminal pancreatic necrosectomy using a self-expanding metal stent and high-flow water-jet system. *World J Gastroenterol* 2013; 19(23): 3685-3692 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3685.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3685

INTRODUCTION

Acute pancreatitis (AP) is a disease characterized by significant morbidity and mortality. Pancreatic necrosis (PN), occurring as diffuse or focal areas of non-viable pancreatic parenchyma as defined by the 1992 Atlanta classification, is a serious complication that can develop within a few days following the onset of AP[1].

Acute necrotizing pancreatitis (ANP) complicates 15%-20% of all cases with AP and, in patients in whom the most severe infectious complication infected necrosis develops, the mortality can be as high as 25%-70%[2].

"Organized" or "walled-off pancreatic necrosis" (WOPN), described as relatively well-circumscribed areas of PN, evolves during the several weeks after an episode of severe ANP. This process reflects the evolution from early diffuse PN to an encapsulated, loculated form[3].

Similarly, a pancreatic abscess (PA), a serious complication of AP, can be defined as a circumscribed intra-abdominal collection of purulent material adjacent to the pancreas, containing little or no PN, but circumscribed within a well-defined wall[1].

The conventional treatment of infected WOPN and PA necessitates open surgical debridement, together with sump drainage and continuous lavage. The key objectives of the surgical approach are the removal of all pancreatic- and peripancreatic necrotic tissue, the evacuation of purulent infected material, and the provision of continuous and adequate drainage to promote resolution of the inflammatory processes. The best current therapeutic approach comprises complete removal of the necrotic material, but preservation of all the viable pancreatic tissue, together with the best possible supportive care, preferably at an intensive care unit (ICU).

The recommended surgical procedure of open necrosectomy is associated with a high postoperative complication rate (95%)[4], and the median mortality rate has been reported to be 25% (range 6%-56%)[5].

Minimally invasive approaches with high success and lower mortality rates are therefore of considerable interest, and various percutaneous and endoscopic techniques for the management of PN and PAs have been described during the last decade.

Transluminal retroperitoneal endoscopy for the debridement of infected WOPN can be considered one of the first clinical applications of natural orifice transluminal endoscopic surgery. However, the history of endoscopic necrosectomy began with the endoscopic transmural drainage of pancreatic pseudocysts and abscesses[3].

Endoscopic interventions are typically performed under conscious sedation without the need for general anesthesia. Endoscopic therapy for PN may involve endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy, stenting, and nasojejunal feeding tube placement, while a more aggressive and potent therapeutic approach is transmural (transgastric or transduodenal) endoscopic drainage[3], with or without endoscopic ultrasonographic guidance[6,7], followed by balloon dilation of the cystogastrostoma or cystoduodenostoma, and repeated direct endoscopic necrosectomies through the dilated fistulous tract using different accessories (*e.g.*, a polypectomy snare, Dormia basket, hot biopsy forceps, or tripod grasper)[8,9], and stent and/or catheter placement into the necrotic cavity, followed by continuous lavage and irrigation[10] and, if required, repeated balloon dilations of the fistulous tract.

Two large, multicenter retrospective studies have demonstrated that the direct transluminal endoscopic management of PN is associated with good long-term maintenance of the high initial success rate[11,12]. Nevertheless, all of the current endoscopic techniques have obvious inherent limitations, such as the risk of air embolism, endoscopically uncontrollable bleeding, and inadequate drainage through multiple plastic stents, together with an early occlusion of the fistulous tract.

To overcome these difficulties, we demonstrate here a new and successful method of endoscopic transluminal necrosectomy (ETN); a combination of the temporary placement of a self-expanding metal stent (SEMS) into the fistulous tract and daily irrigations of the necrotic cavity with a high-flow water-jet system, using a flush knife.

CASE REPORT

Direct endoscopic therapy was performed on 4 patients. The indication for the endoscopic transmural approach was infected PN in 3 cases and a PA in 1 case.

Case 1

A 59-year-old female was admitted to our emergency sub-ICU with AP. At admission, she had severe upper abdominal pain that had started 24 h before. Abdominal ultrasonography revealed multiple small stones in the gallbladder, with concomitant dilation of the extrahepatic bile ducts. Laboratory tests demonstrated leukocytosis (26 g/L), and elevated blood glucose (13.4 mmol/L), liver function tests [aspartate aminotransferase (AST): 76 U/L, alanine aminotransferase (ALT): 106 U/L, gamma-glutamyl transferase (GGT): 389 U/L], and serum amylase (2262 U/L). The calculated Glasgow score was 7, which predicted a severe attack of pancreatitis. Emergency ERCP was performed, and after endoscopic sphincterotomy a small (5 mm in diameter) impacted gallstone was successfully removed from the common bile duct. Despite maximum conservative therapy, an abdominal

computed tomography (CT) scan 72 h later demonstrated severe APN with extensive necrosis and peripancreatic fluid accumulation was observed (Balthazar score: 6). After transmission to the ICU she received maximum supportive care, with mechanical ventilation, parenteral antibiotics (Imipenem and Metronidazole), parenteral volume replacement and nasojejunal feeding, epidural anesthesia and plasmapheresis. Despite the optimum therapy, her general condition was still deteriorating at the end of the third week, due to a high fever, sepsis, and multiorgan failure. Laboratory tests showed elevated C-reactive protein (CRP) and procalcitonin (PCT) levels (226 mg/L and 2.72 μg/L, respectively). A repeated CT scan demonstrated an approximately 14 cm × 11 cm × 12 cm volume of not clearly demarcated peripancreatic necrosis with accumulated fluid located between the stomach and the body of the pancreas; clinically, infection of the necrosis was assumed. After repeated surgical consultations, we decided to attempt ETN rather than open surgical necrosectomy in order to minimize the level of invasiveness in this critically-ill patient on day 21 after the onset of ANP.

Case 2

A 62-year-old female patient was transferred from a secondary care hospital to our gastroenterological sub-ICU with upper abdominal pain and an elevated serum amylase level (4000 U/L). She had experienced the first attack of abdominal pain 2 wk earlier, though she then became asymptomatic, but the day before admission she again developed severe abdominal pain after a fatty food intake. At admission, the calculated Glasgow score was 8, indicating a severe attack of AP. On abdominal ultrasonography a large (2 cm in diameter) solitary gallbladder stone was detected. Laboratory tests demonstrated leukocytosis (21 G/L), elevated blood glucose (21 mmol/L), liver function tests (AST: 439 U/L, ALT: 339 U/L, GGT: 308 U/L), and serum amylase (1152 U/L). Emergency ERCP was performed, but, apart from the known gallbladder stone, no cholangiographic signs of biliary obstruction or gallstone pancreatitis were seen. Two days later, due to high fever and sepsis, she was transferred to the ICU. An abdominal CT scan on day 4 of hospitalization revealed ANP with extensive necrosis, duodenal compression, and an accumulation of peripancreatic fluid 45 mm in width at the bursa omentalis (Balthazar score: 7). Maximum supportive care at the ICU included parenteral antibiotics (Imipenem + Amikacin, followed by Tigecycline and Metronidazole), parenteral volume replacement, nasojejunal feeding, and epidural anesthesia; there was no need for long-term mechanical ventilation. A repeated CT scan demonstrated an 11 cm × 7 cm × 7.5 cm volume of peripancreatic necrosis with the extensive accumulation of fluid mainly between the posterior wall of the stomach and the pancreas. At the end of the third week, she still had high fever, and presented extremely high CRP (326 mg/L) and elevated PCT (2.67 μg/L) levels. From the clinical signs and laboratory results, infection of the PN was evident and therefore, after repeated surgical consultations, we decided to perform ETN on day 20.

Case 3

A 72-year-old female patient was admitted to our department with upper abdominal pain and vomiting that had persisted for 6 d before hospitalization. Her case history included hypertension and a vertebrobasilar stroke 7 years previously. At admission, laboratory tests revealed only mild leukocytosis (10.2 g/L), but high CRP (249.6 mg/L), elevated lactate dehydrogenase (741 U/L) and blood urea nitrogen (14.8 mmol/L), and mildly increased serum amylase (136 U/L). The liver function tests were normal, with the exception of mild GGT elevation (110 U/L). The clinical signs and abdominal ultrasonography (no gallstones or bile duct dilatation) led to a diagnosis of idiopathic AP. Despite maximum supportive care, with parenteral antibiotics (Ciprofloxacin and Metronidazole), parenteral volume replacement, nasojejunal feeding, and epidural anesthesia, an abdominal CT scan 3 wk later demonstrated severe ANP, with complete destruction of the pancreatic tissue, secondary antral and duodenal compression, and a 10 cm wide accumulation of peripancreatic fluid in the retrogastric region, from the liver hilum up to the spleen, but not clearly demarcated from the retroperitoneal tissue (Balthazar score: 9). After repeated surgical consultations, we decided on ETN rather than open surgical necrosectomy in order to minimize the invasiveness in this elderly patient with significant comorbidity, on day 31 after the onset of ANP.

Case 4

A 52-year-old male patient was admitted to our department with upper abdominal pain and nausea. The symptoms had developed after a spicy and fatty food intake. The case history revealed two attacks of AP one year previously, with the consequent development of a pancreatic pseudocyst. At admission, laboratory tests indicated mild leukocytosis (12.2 g/L), CRP (15.2 mg/L), and mildly elevated blood glucose (6.5 mmol/L) levels. Liver function tests were normal, but the serum level of amylase was increased (653 U/L). On abdominal ultrasonography a large (11 cm × 4 cm) pancreatic pseudocyst was seen, without signs of bile duct dilation. A temporary improvement was achieved on supportive care, but on day 7 of hospitalization a sudden deterioration was observed and the patient was transferred to the ICU. An abdominal CT scan revealed a PA (8.5 cm × 5 cm). Despite maximum supportive care with mechanical ventilation, parenteral antibiotics (Imipenem and Metronidazole, followed by Amikacin), parenteral volume replacement, and nasojejunal feeding, no improvement was achieved. After repeated surgical consultations, we decided to perform ETN on day 13 after the onset of AP.

Endoscopic treatment

All patients provided informed consent before each endoscopic intervention. The endoscopic procedures were performed with the patients under conscious sedation

with intravenous benzodiazepine and/or opioid administration. ERCP was carried out when indicated.

The feasibility of endoscopic drainage was assessed by endoscopic ultrasonography with the application of a linear echoendoscope (Fujinon EG-530UT). The optimum drainage site was selected, and vessel interposition was excluded by color-flow Doppler. After infiltration of the gastric wall with 20 mL of diluted adrenaline (1:10000 in saline), the cavity was punctured with a 22-gauge needle for sample collection (for microbiological analysis). Next, the gastric wall was perforated with a cystostome (Wilson-Cook, 60 W, forced coagulation) to access the necrotic cavity, followed by dilatation of the fistulous tract up to 12 F with the outer dilatation catheter of the same device. Contrast medium was then injected to assess the extent and borders of the cavity. After this, a 480 cm long, 0.035 inch diameter guidewire (Tracer Wire Guide; Wilson-Cook) was carefully advanced through the cystostome to form at least two loops inside the cavity, so as to facilitate safe catheter exchange. The puncture site was dilated with a wire-guided biliary balloon up to 10 mm and 1.5 psi (Olympus Endoscopy). Finally, a 6 cm long, 10 mm wide throughout, non-covered, biliary SEMS (Micro-Tech Europe) was positioned and opened inside the fistulous tract in order to make a permanent and wide connection between the necrotic cavity and the stomach. After opening of the SEMS, a large volume of purulent fluid and necrotic material emptied spontaneously into the stomach, and this was further facilitated with continuous flushing and suction *via* the endoscope. We finalized the first stage of the necrosectomy process with the placement of a 12 F nasobiliary pigtail catheter into the necrotic cavity through the SEMS. During the next 5 d, physiological saline was irrigated through this catheter at a flow rate of 100 mL/h to facilitate continuous lavage and optimum drainage of the necrotic material.

We entered the stomach with a double-channel therapeutic gastroscope (Fujinon EG-530D), and removed the SEMS from the necrotic cavity without any difficulty with standard foreign body forceps 5-7 d later. ETN was performed in the following steps: (1) the therapeutic gastroscope (Fujinon EG-530D) was inserted directly into the cavity; (2) with the application of a flush knife (Fujinon Flush Knife), the purulent debris was flushed from the inner cavity wall, using the high-flow water-jet system (Fujinon JW-2 Water Pump). For high-flow irrigation, we used Betadine solution (1:10 dilution in saline); continuous suction was achieved through the wide-channel therapeutic endoscope; (3) the necrotic remnant was removed by simultaneous application of the flush knife, a Dormia basket, and hot biopsy forceps; and (4) finally, placement of a 12 F nasobiliary catheter into the necrotic cavity and a suction drain into the stomach provided continuous lavage between the endoscopic sessions.

ETN and lavage were performed daily until complete evacuation of the necrotic and purulent material (Figures 1-3).

The puncture of the necrotic cavity and placement of the SEMS into the fistulous tract, together with nasopancreatic drainage, was successful in all patients. Access to the cavity was created by an endoscopic puncture into a clearly bulging lesion under endosonographic guidance in 3 cases, and by using a spontaneous perforation of the cyst to the gastrointestinal lumen in Case 4. Transgastric (Cases 1-3) or transduodenal access (Case 4) was chosen in 3 and 1 cases, respectively. After 5-7 days of lavage, the SEMSs were all safely removed. No SEMS-related complications were observed. After SEMS removal, the fistulous tract was sufficiently widely open (without any need for further balloon dilation) to be entered repeatedly with a double working channel therapeutic gastroscope; this allowed permanent access for transgastric necrosectomy during the next 2-3 wk. The average number of daily necrosectomies was 2.6 (range, 2-5). No endotherapy-related complications were observed. ERCP was performed in two patients. After endoscopic management, the Sequential Organ Failure Assessment score and the sepsis improved, the decreased level of consciousness ceased, CRP levels significantly decreased (Figure 4), and a CT scan showed no further evolution of peripancreatic fluid, necrosis, or abscess. All patients, except one (Case 2), were uneventfully and completely cured after endoscopic therapy and medical treatment. Following 6 wk of further basic supportive treatment and jejunal nutrition, they could leave the hospital. The average duration of hospitalization was 63 d (range 48-74 d). The artificial transluminal fistula spontaneously closed within 3 mo.

After a temporary improvement, one patient (Case 2) died 20 d after the first endoscopic intervention, on day 38 of ICU treatment. The autopsy revealed multifocal pulmonary abscesses and severe bilateral pneumonia (an obvious complication of prolonged ventilation), but no retroperitoneal abscess and only moderate inflammatory signs at the site of ETN.

DISCUSSION

Despite receiving maximum ICU therapy, patients with ANP can succumb rapidly. In terms of the clinical disease course, severe AP is biphasic. During the first week after the onset, there is an early mortality peak, which is known to be mainly due to the systemic inflammatory response syndrome and early multiorgan failure evoked by the exaggerated pro-inflammatory response[13,14]. In contrast, 2-3 wk after the onset of the symptoms, at the time of the maximum anti-inflammatory response and consequent immunosuppression, there is a second peak of mortality, which is known to be mainly due to the infectious complications caused by bacterial translocation, followed by sepsis and late multiorgan failure[15,16]. At this point, even maximum ICU therapy may not be able to halt or reverse the disease progression in some patients.

The standard treatment for infected and complicated WOPN is surgical intervention with open necrosectomy and drainage[17,18]. If it is considered that ANP is responsible for the multiple organ dysfunction syndrome, con-

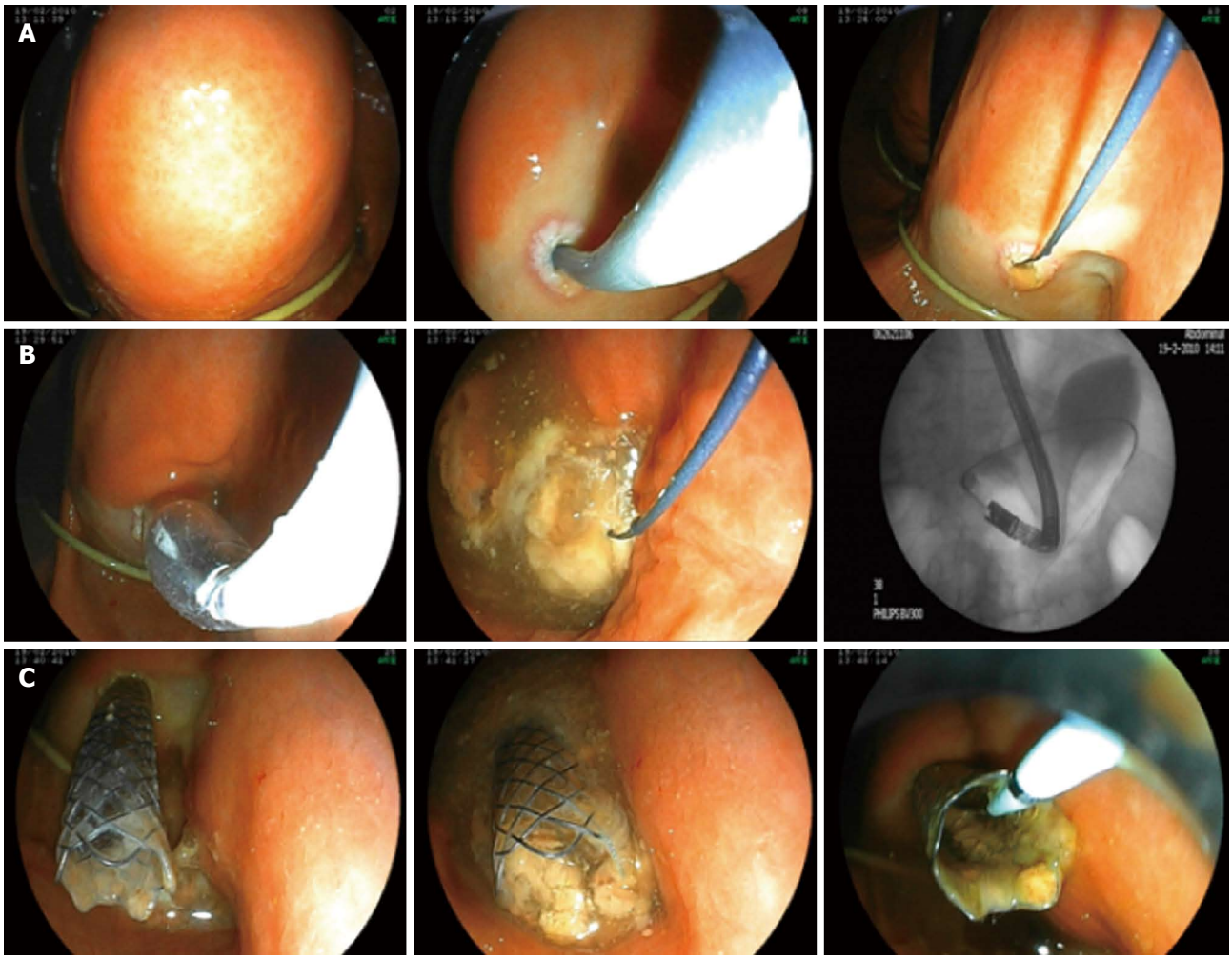

Figure 1 Endoscopic treatment of a large walled off pancreatic necrosis. A: Puncture; B: Dilatation; C: Self-expanding metal stent implantation (Case 1).

ventional early surgery (with its high complication rates) is the possible second hit, which may be the explanation, at least in part, for the high mortality. This concept is compounded by the fact that minimally invasive approaches, including minimally invasive retroperitoneal surgical necrosectomy[19], percutaneous catheter drainage[20], and endoscopic transluminal drainage (ETD)[3] ETN[6-12,21], are thought to induce less physiological stress and result in less activation of the inflammatory processes than equivalent open surgery, and may therefore improve the overall outcome of patients with infected PN.

Transgastric endoscopic stent placement, mainly under endoscopic ultrasonographic guidance, is helpful for the drainage of abscesses, but its success in cases of infected WOPN is rather limited. In 2000, Seifert *et al*[6] first described transgastric access with a standard endoscope into the retroperitoneal space for necrosectomy and debridement. The comparison of ETN with ETD by Gardner *et al*[7] indicated that ETN achieves higher rates of resolution relative to ETD, without concomitant changes in the number of endoscopic procedures, the complication rate, or the time to resolution. To date, numerous workgroups have reported success and convincing results, including the low morbidity and mortality of ETN in the treatment of infected WOPN in case series[8,9,22]. Moreover, two large, multicenter retrospective studies from Germany and the United States have demonstrated that transmural minimally-invasive endoscopic treatment of WOPN is an efficacious and reproducible technique with an acceptable safety profile[11,12]. However, all studies involving transluminal pancreatic necrosectomies are challenged by the limitations in the efficacy of the endoscopic devices and accessories used for debridement (*e.g.*, Dormia baskets and polypectomy snares). Additional novel methods have recently been described that may facilitate debridement during ETN and improve the overall outcome. Belle *et al*[23] showed that, through the temporary use of a special partially-covered SEMS designed to keep the pancreaticogastrostomy open for the drainage of WOPN, clinical success (defined as the complete removal of necrotic masses without major complications) was achieved in all 4 of the study's patients. Furthermore, Antillon *et al*[24] reported one case where placement of a large-diameter removable metallic esophageal stent into the necrotic cavity, in conjunction with intensive lavage facilitated drainage, was effective; when previous multiple sessions of ETN and drainage with plastic stents had

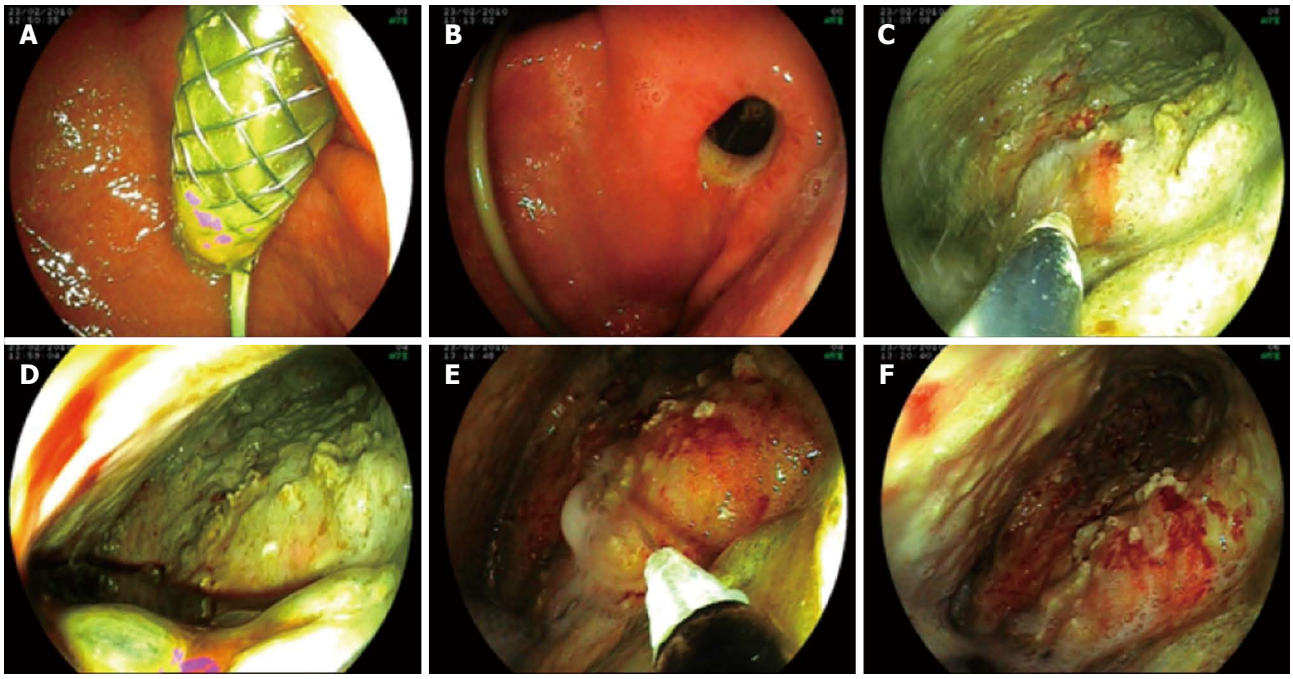

Figure 2 Endoscopic treatment. Removal of self-expanding metal stent (A, B), endoscopic transluminal necrosectomy (C, D), application of high-flow water-jet system using a flush knife (E, F) (Case 2).

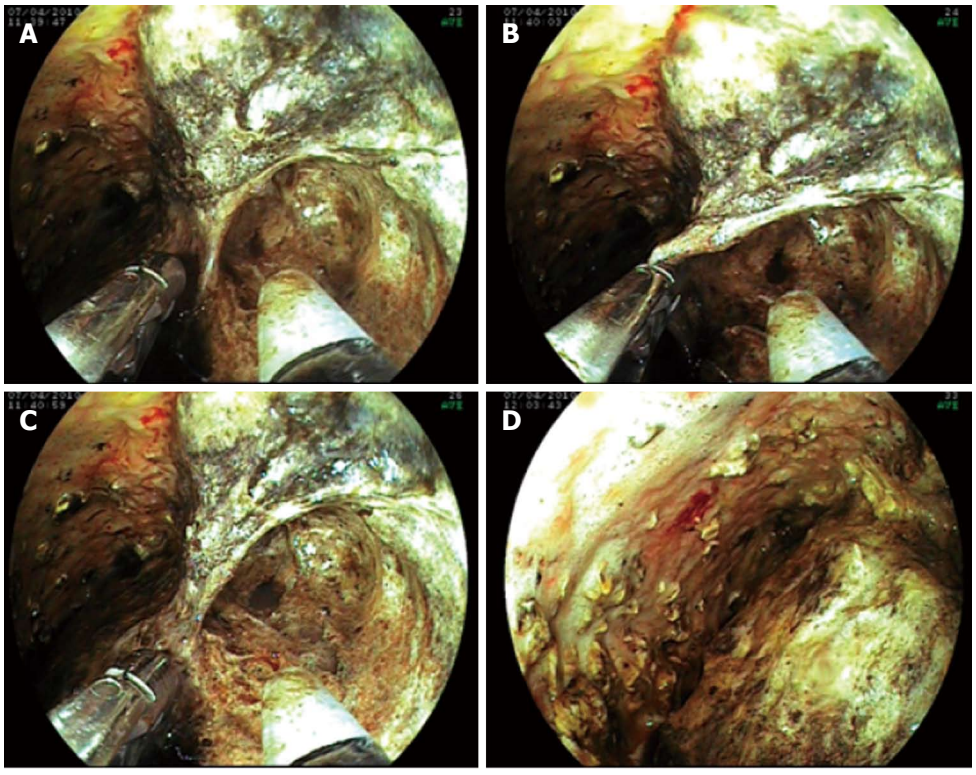

Figure 3 Endoscopic treatment. Endoscopic transluminal necrosectomy (A), application of high-flow water-jet system using a flush knife and hot biopsy forceps (B-D) (Case 3).

failed.

We have presented here a new and highly efficient method of ETN as a combination of the temporary placement of a non-covered biliary SEMS into the fistulous tract and daily irrigations of the pancreatic necrotic cavity during direct necrosectomies with a high-flow water-jet system, using a flush knife.

The temporary placement of the SEMS resulted in a sustained, open fistulous tract with a wide orifice between the stomach and pancreatic necrotic cavity, through

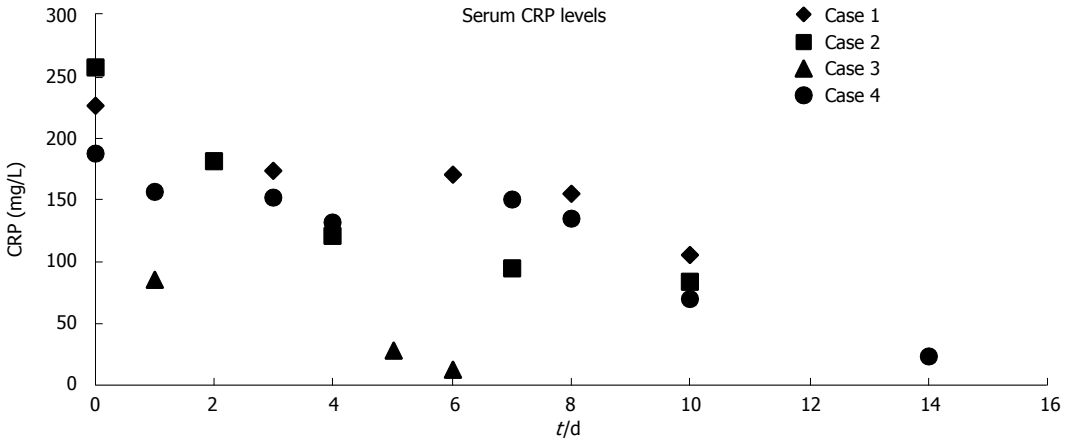

Figure 4 Effect of endoscopic transluminal therapy on C-reactive protein levels (first endoscopic intervention at day 0). CRP: C-reactive protein.

which the ETN could easily be traced and re-performed (even 2 wk after removal) without any additional plastic stent insertion. The relatively short duration in place of the SEMS may prevent stent-related potential complications such as migration, stent-end rubbing-associated cavities, or stomach wall erosions, bleeding or perforation. The necrotic tissue, including large particles, and the purulent material could be removed and/or emptied to the stomach without any complication by washing the cavity with the high-flow water-jet system, using the flush knife, and providing continuous suction *via* the double-channel therapeutic gastroscope. Only for the removal of adhesive necrotic remnants did we use other accessories (a Dormia basket and hot biopsy forceps) to facilitate the debridement. During this effective, but from a surgical aspect relatively non-aggressive approach, we experienced no procedure-related complications.

Earlier, when we have performed ETN, several hours after each direct endoscopic debridement session we observed a temporary temperature increase in the patients (data not shown). It is speculated that this may be associated with the extreme endotoxin challenge resultant from the debris removed from the necrotic cavity to the gastrointestinal tract, and the impaired gastrointestinal barrier function. We hypothesize that continuous lavage performed constantly together with suction may prevent this event.

We also included a patient with a PA (Case 4) which consisted mainly of solid particles and we had therefore decided to perform direct ETN instead of drainage alone.

We believe that this new combination of methods may lead to clinical success as a consequence of its efficacy with fewer complications and an improvement in patient comfort. However, in order to confirm and optimize this endotherapy, further studies are necessary, preferably on a larger patient population.

The endoscopic transluminal management of PN and/or PA is associated with good initial and long-term clinical success, with acceptable morbidity and mortality rates. The advantages of endoscopic management are related to its minimal invasiveness. The combination of multiple endoscopic approaches is designed to achieve the goals of any treatment strategy.

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CASE REPORT

Polyarteritis nodosa clinically mimicking nonocclusive mesenteric ischemia

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Abstract

Here, we present the case of a 74-year-old Japanese man with segmental intestinal necrosis, which developed after treatment with pulsed methylprednisolone for mononeuritis multiplex. The patient was weakly positive for myeloperoxidase (MPO)-anti-neutrophil cytoplasmic antibody (ANCA). Computed tomography and surgical findings were compatible with nonocclusive mesenteric ischemia (NOMI). He underwent small intestinal resection by emergency surgery and an intestinal fistula was made. Pathologically, necrotizing vasculitis with fibrinoid necrosis was present in medium to small-sized arteries, which was equivalent to Arkin's classification Ⅱ-Ⅳ. Most of the arteries had fibrous intimal thickening, which was considered to obstruct the arteries and thus cause segmental intestinal necrosis. A diagnosis of polyarteritis nodosa (PAN) was made, and intravenous cyclophosphamide pulse therapy was added to the therapeutic regimen. This patient was successfully treated with these multidisciplinary therapies and his stoma was finally closed. This is a very rare and indicative case of PAN weakly positive for MPO-ANCA and clinically mimicking NOMI, which occurred even after treatment with pulsed methylprednisolone.

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Key words: Anti-neutrophil cytoplasmic antibody; Intestinal necrosis; Myeloperoxidase; Nonocclusive mesenteric ischemia; Polyarteritis nodosa

Core tip: We present a patient with polyarteritis nodosa (PAN) weakly positive for myeloperoxidase-anti-neutrophil cytoplasmic antibody and clinically mimicking nonocclusive mesenteric ischemia (NOMI), which occurred after treatment with pulsed methylprednisolone for mononeuritis multiplex. The present case is not only rare but also informative, because vasculitis in medium to small-sized arteries was shown to take a few months to develop tangible signs of visceral ischemia, which can occur even after treatment with pulsed methylprednisolone, and the imaging and surgical findings of intestinal necrosis caused by PAN may resemble those of NOMI.

Shirai T, Fujii H, Saito S, Ishii T, Yamaya H, Miyagi S, Sekiguchi S, Kawagishi N, Nose M, Harigae H. Polyarteritis nodosa clinically mimicking nonocclusive mesenteric ischemia. *World J Gastroenterol* 2013; 19(23): 3693-3698 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3693.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3693

INTRODUCTION

Polyarteritis nodosa (PAN) is necrotizing arteritis of medium to small-sized arteries without glomerulonephritis or vasculitis in arterioles, capillaries, or venules[1]. PAN can show a wide variety of symptoms, including general symptoms, neurological manifestations, skin involvement, renal involvement, and gastrointestinal (GI) manifestations[2]. Clinically, the spectrum of GI manifestations is wide, ranging from mild transient abdominal pain to life-threatening complications requiring emergency surgery, *e.g.*, peritonitis, bowel infarction, or hemorrhage[3]. Severe GI involvements, including bowel perforation, bleeding, and pancreatitis, are independent predictive factors for poor prognosis of PAN together with age[4]. Although GI ischemia has been reported to occur at a rate of 13%-31% in PAN patients[3,5], the prevalence of PAN itself is very low, and clinical suspicion of vasculitis is sometimes difficult in cases showing intestinal necrosis. Here, we describe a case in which a patient with PAN presented with segmental intestinal necrosis clinically mimicking nonocclusive mesenteric ischemia (NOMI) even after treatment with pulsed methylprednisolone for vasculitis.

CASE REPORT

A 74-year-old Japanese man was admitted to our hospital because of mononeuritis multiplex in the left ulnar and peroneal nerves on May 26, 2012. Two months previously, he had experienced systemic muscular pain. A tentative diagnosis of polymyalgia rheumatica was made and he was treated with prednisolone (PSL) at 10 mg/d. Although steroids were partially effective in improving the patient's condition, the levels of C-reactive protein (CRP) continued to be high (18 mg/dL). He was referred to our hospital for further evaluation one month later, at which time he had no complaints other than muscular pain. His height was 172 cm and body weight was 62 kg; the patient's body weight had decreased by 3 kg from the onset of the disease. Laboratory tests indicated leukocytosis, anemia, thrombocytosis, and elevated CRP levels. The results of urine tests were negative. The patient was negative for anti-nuclear antibody and proteinase 3 (PR3)-anti-neutrophil cytoplasmic antibody (ANCA), but weakly positive for myeloperoxidase (MPO)-ANCA (23.7 U/mL: normal range 0.0-8.9 U/mL). Tests for infections, including hepatitis B surface antigen and blood culture, were negative. Torso computed tomography (CT) revealed emphysema alone, and whole-body positron emission tomography yielded negative results. Although we recommended hospitalization for diagnosis and treatment, the patient refused for personal reasons. One week later, he noticed numbness in his left hand and leg. He then developed left foot drop, so underwent a medical examination and was admitted to our hospital. On admission, his consciousness was clear, performance status was 3, body temperature was 36.3 ℃, and blood pressure was 178/124 mmHg. He had general muscle weakness and sensory loss in regions supplied by the left ulnar and peroneal nerves, and dorsiflexion of the left foot was compromised to manual muscle testing 2/5. Laboratory tests indicated elevated levels of CRP and muscular enzymes, such as creatine kinase (CK) (Table 1). Urinary analysis showed proteinuria and hematuria with a few casts. He received pulsed methylprednisolone at a dose of 1 g for 3 d followed with PSL at 60 mg/d because the underlying disease was considered to be vasculitis, and intravenous nicardipine for hypertension, which was considered to be caused by renal vascular involvement. Despite this therapy, the CRP level did not fall below 10 mg/dL and the CK level increased significantly to 9358 IU/L after 3 d of this treatment (Figure 1). At the same time, the patient complained of abdominal pain. His abdomen was flat and soft, and showed left lower quadrant pain without muscular defense or rebound. Abdominal X-ray showed a distended intestine, indicating paralytic ileus. CT revealed distended small bowel loops, gas in the small bowel, and blurred enhancement of the intestinal wall without any significant obstruction in the mesenteric arteries, suggesting NOMI (Figure 2A-C). Emergency surgery was performed on the same day. His jejunum to ileum showed segmental ischemia and necrosis over 2 m, and surgical findings were compatible with NOMI (Figure 2D). Small intestinal resection was performed and an intestinal fistula was made. Pathologically, necrotizing vasculitis with fibrinoid necrosis was present in medium to small-sized arteries, and most of the arteries had fibrous intimal thickening (Figure 3). There was no necrotizing vasculitis in arterioles, capillaries, or venules. Pathology was equivalent to Arkin's classification Ⅱ-Ⅳ[6], and a diagnosis of PAN was made. Intravenous cyclophosphamide (IVCY) pulse therapy at a dose of 500 mg/mo was added to the treatment regimen. Although various complications occurred, including deep vein thrombosis, stoma trouble, and disseminated varicella zoster virus infection, the patient recovered well and was transferred to a different hospital for rehabilitation. As his nutritional status had improved because he became able to eat a regular diet without supplemental nutrition, his stoma was closed in April 2013. He is now in complete remission with the dose of PSL tapered to 10 mg/d.

DISCUSSION

NOMI is an acute mesenteric circulatory disorder that is not caused by organic occlusion of blood vessels[7]. The typical patient is critically ill, with severe cardiac disease or sepsis[8]. With regard to pathogenesis, intestinal vasospasm due to persistent low perfusion is thought to cause ischemic disorder due to decreased cardiac output and blood pressure[9]. Mitsuyoshi *et al*[7] reported findings that may be useful as supplemental information in diagnosis of NOMI: (1) enhancement of principal arteries could be traced to the periphery close to the marginal arteries in CT slices; and (2) the staining intensity varied in the intestinal wall in the same slice (showing a difference between regions of the intestinal wall with good and poor

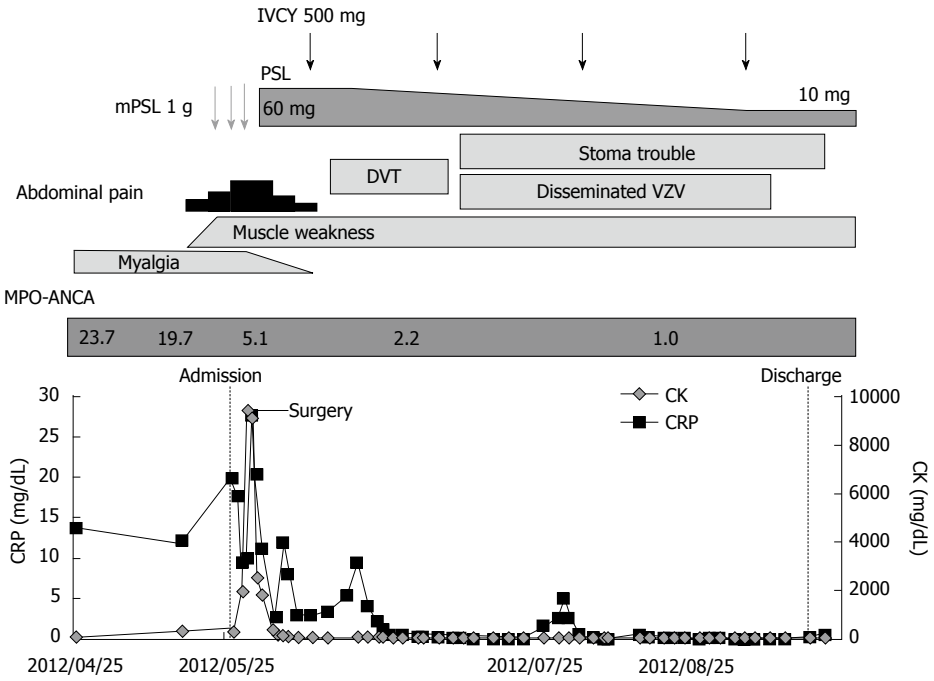

Figure 1 Clinical course. CK: Creatinine kinase; CRP: C-reactive protein; DVT: Deep venous thrombosis; IVCY: Intravenous cyclophosphamide; mPSL: Methylprednisolone; MPO-ANCA: Myeloperoxidase-anti-neutrophil cytoplasmic antibody; PSL: Prednisolone; VZV: Varicella zoster virus.

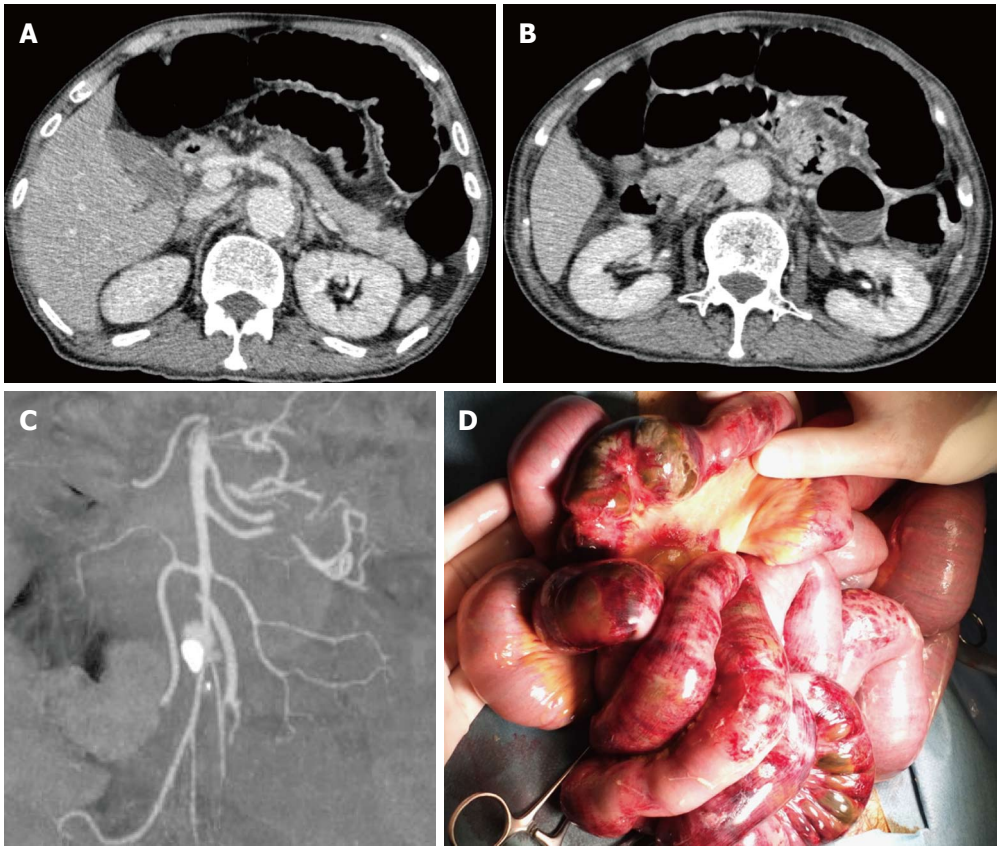

Figure 2 Clinical imaging. A: Computed tomography (CT) image of the abdomen showing distended small bowel loops, gas in the small bowel, blurred enhancement of the intestinal wall, and absence of any significant obstruction in the celiac trunk; B: CT image of the abdomen showing superior mesenteric artery; C: CT angiographic reconstruction of the superior mesenteric artery; D: Surgical findings showing distended small intestine, segmental intestinal ischemia, and necrosis.

blood flow). With regard to laparotomic characteristics, mesenteric blood flow may be retained, even in marginal arteries reaching the lesions, despite extensive necrotization throughout the intestine (noncontinuous segmental necrosis). This was reported to be a marked distinguishing feature from mesenteric thrombosis, in which the mesentery and intestine are necrotized from the site of the thrombus, forming a sphenoidal necrotic area in the region served by the artery (continuous necrosis)[7].

Although CT imaging and surgical findings were compatible with NOMI in this case, the underlying disease was considered to be vasculitis because this patient manifested general weakness, myalgia, and mononeuritis multiplex; clinically apparent mononeuritis multiplex can be a diagnostic or classification criterion for vasculitis affecting peripheral nerves without the need for a nerve

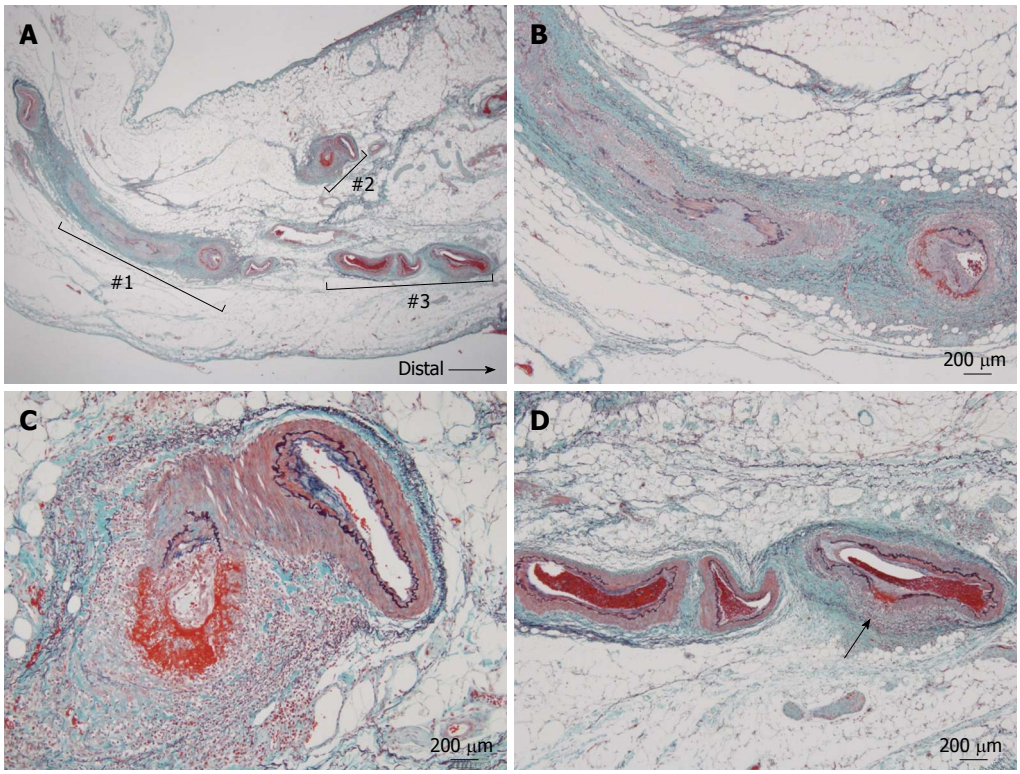

Figure 3 Histological findings of the jejunum. A: Necrotizing vasculitis of the mesenteric artery showing nodular lesions in various stages of the Arkin classification, Elastica-Masson staining; B: Higher magnification of No. 1 in A showing necrotizing vasculitis of mesenteric artery in stage Ⅱ-Ⅲ; C: Higher magnification of No. 2 in A showing necrotizing vasculitis of the mesenteric artery in stage Ⅱ and panarterial necrosis with fibrinoid degeneration; D: Higher magnification of No. 3 in A showing necrotizing vasculitis of mesenteric artery in stage Ⅱ, characterized by destruction of internal elastic lamina associated with fibrinoid necrosis (arrow).

biopsy to observe the vasculitis histologically[10]. In this case, necrotizing vasculitis with fibrinoid necrosis was pathologically present in medium to small-sized arteries, and fibrous intimal thickening was considered to obstruct these arteries and cause segmental intestinal necrosis. Although the pathology was equivalent to Arkin's classification Ⅱ-Ⅳ, most belonged to Arkin's classification Ⅲ-Ⅳ, indicating longstanding vasculitis. Therefore, it is reasonable to consider that the patient suffered from vasculitis from the beginning. Although he was treated with pulsed methylprednisolone before the onset of abdominal symptoms, fibrous intimal thickening of medium to small-sized intestinal arteries must have already occurred.

According to the Chapel Hill Consensus Conference 2012[10], PAN is defined as necrotizing arteritis of medium to small-sized arteries without glomerulonephritis or vasculitis in arterioles, capillaries, or venules, and is not associated with ANCA, which was based on the report that ANCA was typically absent in patients with PAN[11]. In this previous report by Guillevin *et al*[11], one patient (Table 1, No. 40) diagnosed with PAN had MPO-ANCA. Furthermore, there have been some reports presenting pathologically diagnosed PAN with MPO-ANCA positivity, all of which were reported from Japan[12-16]. The prevalence rates of ANCA and ANCA-associated vasculitis are different between Japan and Europe; microscopic polyangiitis (MPA) and MPO-ANCA are more common in Japan, while granulomatosis with polyangiitis and PR3-ANCA are more common in Europe[17,18]. These trends may be related to the reports of PAN with MPO-ANCA positivity in Japan.

Although MPO-ANCA was also weakly positive in this case, this patient clearly fulfilled the American College of Rheumatology 1990 criteria for classification of PAN[19], the pathological findings were typical of PAN as shown in Figure 3, and vasculitis was absent in arterioles, capillaries, and venules. Therefore, a diagnosis of PAN was made in this case. Tanaka *et al*[14] described a case of PAN with MPO-ANCA and vasculitis in mesenteric medium-sized arteries, which was confirmed by autopsy. In their case, the titer of MPO-ANCA was low and was not correlated with the severity of PAN. These points were similar to our case, and we speculated that the epitope and pathogenicity may be different from those of MPO-ANCA found in MPA. Peripheral nervous system vasculitis, hypertension, cutaneous lesions, and myalgias were reported to be more common in PAN with than without GI involvement[5], and our patient also manifested most of these symptoms with the exception of cutaneous lesions.

The standard regimen for PAN, not related to hepatitis B virus infection, is based on a combination of corticosteroids (CS) and CY[20]. The addition of CY to CS particularly benefits patients presenting with factors associated with poor prognosis, such as GI involvement. Intermittent pulse-therapy may be as efficacious as oral CY for inducing remission, while generating fewer side effects. Treating PAN patients positive for factors associated with poor prognosis with 12 rather than 6 CY pulses significantly decreased the relapse rate and significantly increased the probability of event-free survival[20]. Plasma exchange can be prescribed for severe life-threatening PAN as combined rescue therapy, although trials have not proven its benefits when prescribed systematically for all patients with PAN[21].

In addition, the surgical management of patients with acute abdominal syndromes has also improved, and now

Table 1 Laboratory findings

	May 2012	Pre-surgery	Post-surgery	Mar 2013
Urinalysis				
Protein	1+	2+	1+	-
Occult blood	3+	3+	3+	-
Red blood cell	5-9/HPF	5-9/HPF	5-9/HPF	-
Casts	+	+	+	-
Granular	< 4/HPF	< 4/HPF	< 4/HPF	
WBC (/μL)	23700	40700	20800	9500
Segmented cells	89%	87%	93%	56%
Band cells	0%	6%	0%	0%
Eosinophils	3%	0%	0%	1%
Basophils	0%	0%	0%	0%
Lymphocytes	6%	2%	5%	33%
Monocytes	2%	5%	2%	10%
Hb (g/dL)	11.5	11.4	8.4	10.1
PLT (10^4/μL)	49.1	32.8	22.7	24.1
T-Bil (mg/dL)	0.6	1.1	0.8	1.3
AST (IU/L)	39	352	89	36
ALT (IU/L)	32	181	107	45
LDH (IU/L)	304	641	427	217
ALP (IU/L)	368	403	301	326
CK (IU/L)	374	9063	1796	23
TP (g/dL)	6.0	5.6	5.3	5.1
Alb (g/dL)	2.1	2.1	2.6	3.0
BUN (mg/dL)	40	60	52	18
Cr (mg/dL)	0.98	1.11	1.02	1.00
CRP (mg/dL)	19.7	27.6	11.0	0.1
MPO-ANCA (U/mL)	19.2	5.1		Negative

HPF: High-power field; WBC: White blood cells; Hb: Hemoglobin; PLT: Platelets; T-Bil: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; ALP: Alkaline phosphatase; CK: Creatine kinase; TP: Total protein; Alb: Albumin; BUN: Blood urea nitrogen; Cr: Creatinine; CRP: C-reactive protein; MPO: Myeloperoxidase; ANCA: Anti-neutrophil cytoplasmic antibody.

includes more aggressive surgical management, bowel rest, parenteral nutrition, intensive care unit support, and better wound care[5]. This patient was successfully treated with these multidisciplinary therapies, including emergency surgery followed by PSL and IVCY against PAN. Although small intestinal stoma, central venous catheter, sub nutrition, and immunosuppressive therapy caused many life-threatening complications, close monitoring and appropriate treatment resulted in complete remission of PAN and eventual closure of his stoma.

In conclusion, vasculitis in medium to small-sized arteries takes a few months to show tangible signs of visceral ischemia, and the CT and surgical findings of intestinal necrosis caused by PAN may resemble those of NOMI. Clinical awareness of vasculitis and ANCA measurement (never exclusive) are important in managing patients showing segmental intestinal necrosis.

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CASE REPORT

Esophageal mucosal metastasis from adenocarcinoma of the distal stomach

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Abstract

Dissemination of gastric cancer may usually occur by direct spread through the perigastric tissues to adjacent organ, lymphatic spread, and hematogenous spread. We report a rare case of gastric cancer with mucosal metastastic lesion on the upper esophagus that was diagnosed by endoscopy and endosonography. A biopsy of the esophageal mass was performed and the pathologic findings with immunohistochemical stain for Mucin-5AC are proved to be identical to that of gastric adenocarcinoma, suggesting metastasis from main lesion of the gastric cancer. The lesion could not be explained by lymphatic or hematogenous spread, and its metastasis mechanism is considered to be different from previous studies. We suggest that the gastroesophageal reflux of cancer cells could be one of the possible metastatic pathways for metastasis of esophagus from an adenocarcinoma of the stomach.

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Key words: Stomach cancer; Neoplasm metastasis; Esophagus

Core tip: I believe the paper may be of particular interest to your readers because the reason is as follows. First, there has been rarity of case reports about esophageal metastasis from gastric cancer without any evidence of lymphatic involvement or direct spread from the primary lesion. Second, gastroesophageal reflux of cancer cells could be one of the possible metastatic pathways for metastasis of esophagus from an adenocarcinoma of the stomach, and this case proves the possibility of direct implantation of gastric adenocarcinoma cells refluxed on esophagus.

Ki SH, Jeong S, Park IS, Lee DH, Lee JI, Kwon KS, Kim HG, Shin YW. Esophageal mucosal metastasis from adenocarcinoma of the distal stomach. *World J Gastroenterol* 2013; 19(23): 3699-3702 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3699.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3699

INTRODUCTION

Dissemination of gastric cancer may usually occur through one of following three pathways: (1) direct spread through the perigastric tissues to adjacent organ; (2) lymphatic spread; and (3) hematogenous spread[1,2].

Herein we report a rare case of gastric cancer with

mucosal metastastic lesion on the upper esophagus that was diagnosed by endoscopy and endosonography. The lesion could not be explained by lymphatic or hematogenous spread, and its metastasis mechanism is considered to be different from previous ones.

CASE REPORT

A 60-year-old man was admitted to our institution for systemic chemotherapy. Fourteen months prior to the admission, he was diagnosed with advanced gastric carcinoma on the antrum (Figure 1A). Palliative subtotal gastrectomy was performed with gastrojejunostomy to relieve pyloric obstruction, and the pathologic finding of surgically resected stomach disclosed adenocarcinoma.

Follow-up abdominal computer tomography was done a week prior to the current admission which revealed multiple hepatic metastases. The patient complained of dysphagia, and therefore endoscopy was performed. The endoscopic examination of the upper digestive tract showed a single, 1 cm-sized, polypoid mass which located 26 cm below the upper incisor (Figure 1B). Evidence of tumor recurrence in remnant stomach was not found. A biopsy of the polypoid mass of esophagus was performed and the pathologic findings with immunohistochemical stain for Mucin-5AC are proved to be identical to that of gastric adenocarcinoma, suggesting metastasis from main lesion of the gastric cancer (Figure 2). Endoscopic ultrasonography (EUS) with a miniature probe of 20 mHz frequency revealed hypoechoic wall thickening of upper esophagus, confined only to mucosal layer without any lymph node enlargement around esophagus (Figure 3).

He was treated with second line of systemic chemotherapy, consisted of docetaxel and cisplatin. However the disease progressed even after 3 cycles of the chemotherapy.

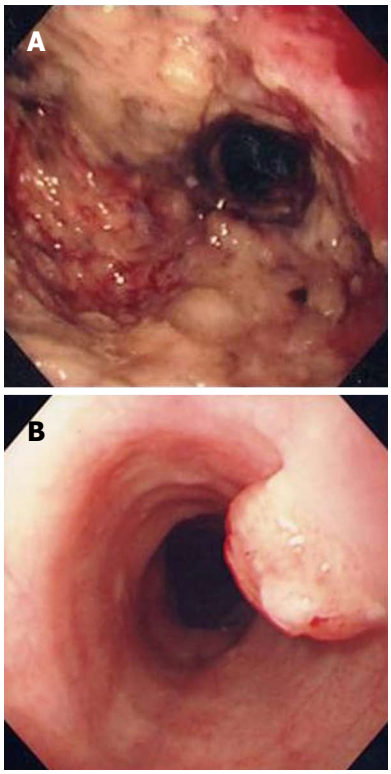

Figure 1 Endoscopic findings. A: Initial endoscopic finding: On the antrum, huge ulceroinfiltrative lesion with irregular margin and uneven dirty base was noted; B: Endoscopic findings 6 mo later: a 1 cm-sized polypoid mass was appeared at 26 cm below the upper incisor.

DISCUSSION

Intramural spread of upper gastrointestinal tract tumors usually occurs *via* abundant lymphatic channels within the submucosal and subserosal layers of the gastric channel and prominent submucosal lymphatic plexus in esophagus. Regardless of the histologic type of the tumor, the tumor is able to infiltrate into submucosal or subserosal layer and spread to adjacent organ *via* lymphatic communication between stomach and esophagus[3-5]. It is considered that esophageal metastasis from the gastric cancers would also be seen as submucosal tumor in gross appearance since it shares same lymphatic channel. In our patient, follow-up endoscopy revealed polypoid mass in upper esophagus instead of appearance of submucosal tumor. EUS of the esophagus performed for assessment of the infiltration depth of the metastatic tumor evidently showed that the tumor was confined to mucosal layer and there was no disruption of muscularis mucosa or enlarged lymph nodes. If there was a pathologic confirmation such as endoscopic submucosal dissection or esophagectomy it must have been definite that the esophageal tumor was confined to mucosal layer. But the resection could not be performed, considering his performance is poor and the disease is markedly progressed. EUS is currently the most accurate means available for tumor staging and locoregional nodal staging[6,7]. Therefore we could conclude that the esophageal tumor was confined to mucosal layer using by EUS.

There has been a case report of gastric cancer with esophageal metastasis which showed very similar finding of esophagus in EUS[8]. The wall of the esophagus at the level of the polypoid lesion was hypo-echoic and thick due to thickened mucosa. In this case total gastrectomy and esophagectomy was performed and the esophageal polypoid lesion was proved to be adenocarcinoma, identical to the primary gastric cancer. In this case report the author speculated that esophageal implantation metastasis from the gastric adenocarcinoma might have taken place by the gastro-esophageal reflux since gastro-esophageal reflux has been documented in various numbers of patients after distal gastrectomy.

Symptoms of gastroesophageal reflux disease have been previously reported to occur in about 30% of patients undergoing distal gastrectomy with Billoth I reconstruction[9]. Distal gastrectomy with Billoth I reconstruction causes two anatomical changes which promote gastroesophageal reflux; the presence of abnormal find-

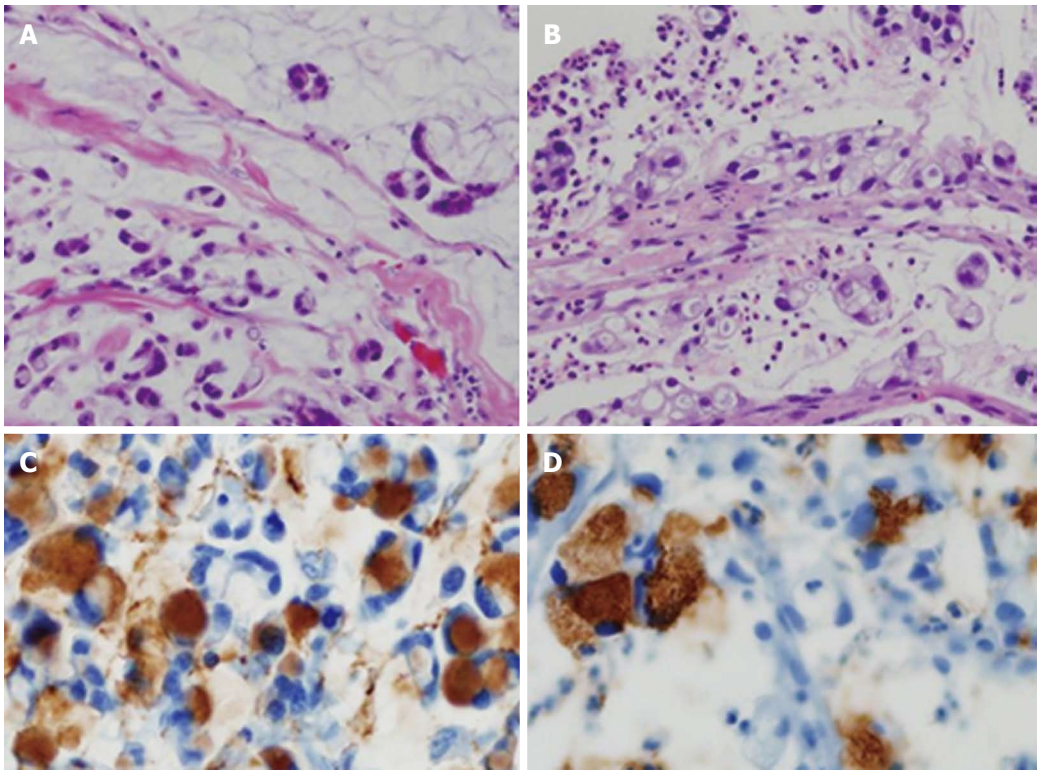

Figure 2 Light microscopic findings. Specimens of stomach (A) and esophagus (B) revealed chains and irregular clusters of tumor cells floating freely in mucous lakes with scattered signet-ring cells [hematoxylin and eosin (HE), × 200]. Mucin-5AC (HE, × 400) is positive in the intracytoplasmic mucin of signet-ring cells of both stomach (C) and esophagus (D) in immunohistochemical staining.

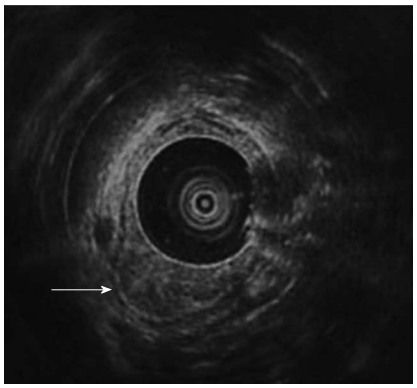

Figure 3 Endoscopic ultrasonographic finding. Hypoechoic wall thickening of esophagus (arrow) was confined to mucosal layer.

ings in the cardia affected by the enlarged angle of Hiss and the high positioning of the remnant stomach in the supine position[9]. This patient underwent palliative subtotal gastrectomy and it is most likely that he had at least gastroesophageal reflux due to the anatomical alterations after surgery and it could have affected the direct implantation of gastric cancer cells on the esophagus.

According to previous study in adenocarcinoma of gastric cancer six patients among a total of 143 patients were verified to have intramural esophageal metastasis[10]. Most of these metastases would have been mediated by lymphatic channels between stomach and esophagus but few could have been done by direct implantation of tumor cells. All patients had gastric cancer from cardia with lymphatic invasion. The distance from the primary tumor of the metastases was 20-50 mm. The metastases were appeared as multiple small submucosal tumors with intact mucosa in some patients. Most of these metastases would have been mediated by lymphatic channels between stomach and esophagus but few could have been done by direct implantation of tumor cells.

In our case, previous multiple hepatic metastases can arouse another possible mechanism of esophageal metastasis, but the esophageal lesion could not be explained by lymphatic or hematogenous spread, and its metastasis mechanism is considered to be different from previous studies.

In hematogenous or lymphatic spread, the esophageal metastasis involves submucosa and usually present as multiple masses, but in our case, esophageal metastasis was single solitary mass and was confined only to mucosal layer without any lymph node enlargement around esophagus. Most intramural esophageal metastases from gastric cancer originate from gastric cardia *via* lymphatic channels. But in our case, gastric cancer had occurred from antrum that was not close to esophagus and esophageal tumor was located at mid esophagus, far from stomach. It was difficult to metastasize from gastric antrum to mid esophagus without adjacent invasion if it metastasized *via* lymphatic channels.

We confirmed that the esophageal mass was metastasized from gastric cancer by pathology using immuno-

histochemical stain. We suggest that the gastroesophageal reflux of cancer cells could be one of the possible metastatic pathways for metastasis of esophagus from an adenocarcinoma of the stomach.

There has been rarity of case reports about esophageal metastasis from gastric cancer without any evidence of lymphatic involvement or direct spread from the primary lesion.

We suggest that the gastroesophageal reflux of cancer cells could be one of the possible metastatic pathways for metastasis of esophagus from an adenocarcinoma of the stomach, and this case proves the possibility of direct implantation of gastric adenocarcinoma cells refluxed on esophagus.

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CASE REPORT

Gastric body diaphragm-like stricture as a rare complication of nonsteroidal anti-inflammatory drugs

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Abstract

Increased risk due to nonsteroidal anti-inflammatory drugs (NSAIDs) therapy has been observed in patients. Although diaphragm-like stricture in the small bowel and colon induced by NSAIDs therapy has been rarely reported, gastric body diaphragm-like stricture has not been reported. We describe the first case of gastric body diaphragm-like stricture due to NSAIDs in a 44-year-old male patient who was successfully treated by an endoscopic approach to avoid complicated surgery. This case highlights new insight into the disadvantages of NSAIDs and provides new data for future clinical studies.

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Key words: Gastric; Gastroscopy; Diaphragm; Stricture; Nonsteroidal anti-inflammatory drug

Core tip: The major disadvantage of nonsteroidal anti-inflammatory drugs (NSAIDs) therapy is the potential to induce adverse gastrointestinal effects. However, diaphragm disease is a rare complication of long-term NSAIDs use. In this study, the first case of NSAIDs-induced diaphragm-like stricture in the gastric body is reported which was successfully treated by an endoscopic approach to avoid a complicated surgical intervention.

Wu LL, Yang YS, Cai FC, Wang SF. Gastric body diaphragm-like stricture as a rare complication of nonsteroidal anti-inflammatory drugs. *World J Gastroenterol* 2013; 19(23): 3703-3706 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3703.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3703

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are known to cause erosion, ulceration, occult bleeding and subsequent stricture formation in the gastrointestinal tract. A rare NSAIDs-induced complication is the formation of diaphragm-like strictures in the intestine[1-4]. Herein, we report, to our knowledge, the first case of NSAIDs-induced diaphragm-like stricture in the gastric body successfully treated by an endoscopic approach to avoid a complicated surgical intervention.

CASE REPORT

A 44-year-old male patient presented with a 2-mo history of abdominal distention and vomiting. There was temporary relief after vomiting and the vomitus was composed of gastric contents. The patient had taken more than 15 g of compound aminopyrine phenacetin tablets by mistake whilst drunk three months previously. On physical examination, his abdomen was tender without any other relevant physical findings. Routine blood and biochemical tests were normal, and tumor markers were within the normal ranges. Gastroscopy showed multiple erosions, ulcers and nodular changes in the proximal gastric body, in which the largest ulcer was

about 1.0 cm × 1.5 cm (Figure 1A, black arrow). Deformation and stricture of the gastric body was noted, and the gastroscope was unable to pass through the stricture (Figure 1A, white arrow). Biopsy pathology at the ulcer edges revealed inflammation and other benign changes. Organic iodine solution radiography of the upper gastrointestinal tract showed a very thin stricture in the gastric body (Figure 1B). Subsequently, a pediatric gastroscope was used, but also failed to pass through the stricture. Repeat biopsy pathology confirmed the previous histology of benign inflammation, and immunohistochemistry showed weakly positive results for CD3, CD43, CD20, Ki-67 and CK, which were consistent with the drug-induced gastric body benign ulcers and stricture. Endoscopic transcatheter radiography was then performed which demonstrated that the gastric body stricture was a diaphragm-like stricture (Figure 1C). The patient underwent balloon dilation and placement of a metal stent (Figure 1D); his symptoms resolved after these endoscopic procedures. On follow-up 2 mo later, the patient had no symptoms.

DISCUSSION

NSAIDs, such as aspirin, indomethacin, diclofenac, and compound aminopyrine phenacetin, are the most commonly prescribed drugs for inflammation, arthritis and cardiovascular protection. However, a major disadvantage of NSAIDs therapy is the potential to induce adverse gastrointestinal effects, particularly in the stomach and duodenum. It has been reported that as many as 25% of chronic NSAIDs users may develop ulcer disease, and 2%-4% of these ulcers may bleed or perforate[5,6]. NSAIDs are absorbed into enterocytes and then uncouple mitochondrial oxidative phosphorylation, resulting in the dysfunction of tight intracellular junctions and intestinal permeability. Enterocytes are thereby exposed to luminal aggressive contents, leading to inflammation and ulceration[7]. Recent clinical research shed light on NSAID-induced small intestinal mucosal damage including erosions and ulcerations, which occur more often than previously expected[8]. Graham *et al*[9] reported that 70% of patients who took NSAIDs for > 3 mo had small intestinal ulcers and erosions shown by capsule endoscopy.

Diaphragm disease induced by NSAIDs, first described by Lang *et al*[1] in 1988, is a rare and severe complication of long-term NSAIDs use, especially in elderly patients[2]. Although diaphragm-like stricture of the small bowel was not associated with the use of NSAIDs in recent reports[10], diaphragm disease was thought at one time to be a unique form of intestinal pathology associated with NSAIDs administration. Disease may occur in 2% of NSAIDs users in the small bowel[3], commonly in the ileum[2,4], or in the duodenum in some cases[11,12]. In the past decade, diaphragm-like strictures in the large intestine due to adverse effects of NSAIDs have been

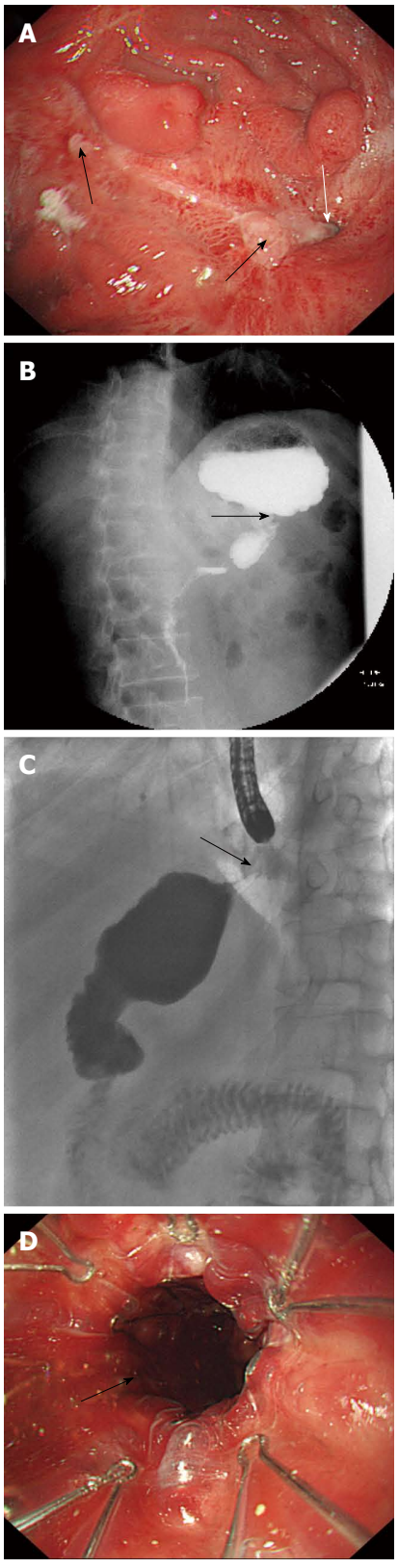

Figure 1 Imaging features of diaphragm-like stricture. A: Gastroscopy showed multiple erosions, ulcers, and nodular changes in the proximal gastric body (black arrow) with a very thin stricture (white arrow) which the gastroscope was unable to pass through; B: Organic iodine solution radiography of the upper gastrointestinal tract showed a tight stricture in the gastric body (arrow); C: Endoscopic transcatheter radiography demonstrated a diaphragm-like stricture (arrow); D: After balloon dilation placement of a metallic stent was undertaken (arrow).

increasingly reported[13,14].

The precise pathogenesis of diaphragm disease is unclear, however, the main histological abnormalities include thickening and chaotic arrangement of muscular bundles in the muscularis mucosae, fibrosis of the lamina propriae and mucosal ulceration[15]. Therefore, affected patients frequently present with gastrointestinal obstructive symptoms and often require surgical treatment.

Although diaphragm-like strictures have been reported in the small bowel and colon, strictures in the gastric body have not been documented in the literature. This may be because the gastric body not only has a wider lumen or space, but also has a thick muscularis. We recently experienced a rare case which occurred after ingestion of a large quantity of NSAIDs. It is known that endoscopic dilation, surgical resection and suspension of NSAIDs administration are common treatment options depending on the position, length and severity of the stricture. In the present middle-age patient, we successfully used the minimally invasive treatment modalities endoscopic dilation and placement of a metal stent, which avoided complicated surgical management.

More and more cases induced by NSAIDs have been reported in the literature, including gastrocolic fistula[16], Brar *et al*[17] reported a case of perforation, and even a case of Crohn's disease was reported which was endoscopically and histologically misinterpreted[18]. Due to the increasing world-wide use of NSAIDs, NSAID-related gastrointestinal complications still continue to be a major concern and require more therapeutic strategies[19-24]. This study, for the first time, reports a rare case of diaphragm-like gastric body stricture which was successfully treated by an endoscopic approach. The endoscope is a useful tool for the diagnosis and treatment of suspected NSAIDs-related gastrointestinal complications.

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CASE REPORT

Ileocecal endometriosis and a diagnosis dilemma: A case report and literature review

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Abstract

Bowel endometriosis affects between 3.8% and 37% of women with endometriosis. The evaluation of symptoms and clinical examination are inadequate for an accurate diagnosis of intestinal endometriosis. We describe the case of a 41-year-old woman who presented to our hospital because of six months of recurrent abdominal pain, vomiting and diarrhea, without previous history of bowel disease. Physical examination revealed a palpable 3 cm × 5 cm mass in the right lower quadrant abdomen. Laboratory tests showed slightly elevated levels of CA19-9 and CA125. Small bowel computer tomography scanning revealed an ileocecal mass with bowel wall thickening and luminal narrowing. Small bowel endoscopy identified a deep longitudinal ulcer and mucosal edema in the distal ileum. All these findings supported the diagnosis of Crohn's disease. The patient underwent a laparotomy, which identified a 5 cm × 5 cm ileocecal mass with severe mucosal edema and luminal stricture in the distal ileum. Histopathological examination confirmed a diagnosis of ileocecal endometriosis without other areas involved. After one-year follow-up, there was no recurrence of the symptoms.

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Key words: Ileus; Bowel obstruction; Longitudinal ulcer; Crohn's disease; Endometriosis

Core tip: We describe the case of a 41-year old woman who had recurrent abdominal pain with vomiting and diarrhea on presentation to our hospital. The results of computer tomography scanning and small bowel endoscopy were strongly suspicious for Crohn's disease. However, surgery and histopathological examination confirmed a diagnosis of ileocecal endometriosis without other areas involved.

Tong YL, Chen Y, Zhu SY. Ileocecal endometriosis and a diagnosis dilemma: A case report and literature review. *World J Gastroenterol* 2013; 19(23): 3707-3710 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3707.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3707

INTRODUCTION

Intestinal endometriosis affects 12%-15% of menstruating women, and is generally an asymptomatic condition[1]. Ileal involvement is very rare and the patients generally present with an asymptomatic or painful mass[2]. Symptoms of bowel endometriosis are numerous, ranging from being asymptomatic to a constellation of symptoms like painful bowel movements, cramps, constipation, diarrhea, vomiting, rectal pain, infertility, abdominal mass, increased urinary frequency and cyclical hematochezia[3].

Classically, the symptoms become worse during menses, but this is not always the case. This myriad of symptoms makes the condition difficult to diagnose. Small bowel endometriosis tends to affect bowel serosa and only 10% of intestinal cases have mucosal involvement[1,4,5]. It can be difficult to discern between ileal Crohn's disease (CD) and endometriosis.

In this report, we describe a further case of ileocecal mass with longitudinal ulcer which was suspected as being CD. Surgery and histopathological examination confirmed a diagnosis of ileocecal endometriosis with no other areas involved. This report serves as a reminder of this rare condition as well as highlighting the diagnostic difficulties it can pose.

CASE REPORT

A 41-year-old lady with no significant past medical history was presented to our hospital because of six months of recurrent abdominal pain, vomiting and diarrhea. The patient had no past history of tuberculosis or other infectious diseases. She also denied radiation exposure, a poisonous chemical contact history and genetic history. Her family had no history of bowel disease. Physical examination revealed a palpable 3 cm × 1.5 cm mass in the right lower quadrant abdomen.

Laboratory tests showed slightly elevated levels of C-reactive protein, CA19-9 and CA125. No other abnormalities were found in tests such as erythrocyte sedimentation rate, immune index or tuberculosis series check. Routine stool test was normal with occult blood negative.

Computer tomography (CT) scanning revealed an ileocecal mass with multiple mesenteric lymph nodes enlarged. A colonoscopy she underwent three months previously showed introverted mucosa surrounding the appendix hole without colon abnormalities. Small bowel endoscopy identified a deep longitudinal ulcer in the distal ileum, mucosal edema and luminal stricture which the endoscope couldn't go through (Figure 1). All these findings supported the diagnosis of CD. To evaluate other intestinal lesions, we undertook small bowel CT scanning, which revealed an ileocecal mass with bowel wall thickening and luminal narrowing, without other intestinal areas being involved (Figure 2).

Due to the bowel obstruction, the patients underwent a laparotomy, which revealed an ileocecal mass, 5 cm × 5 cm in size, with severe mucosal edema and luminal stricture in the distal ileum. No other organs were invaded. The frozen-section diagnosis was endometriosis. En bloc resection was taken and the histopathological examination confirmed ileocecal endometriosis. No subsequent medical treatment was undertaken. The patient recovered well after the surgery, and her quality of life has been significantly improved. After one-year follow-up, there was no recurrence of the symptoms.

DISCUSSION

Endometriosis is defined as the presence of ectopic endometrial tissue in extrauterine sites. It affects 10%-15% of women of reproductive age and usually becomes apparent in the reproductive years when the lesions are stimulated by ovarian hormones[6]. Intestinal endometriosis occurs in 12%-15% of cases, and the incidence of the involvement of different intestinal sites varies greatly in the literature, with the rectosigmoid colon, small bowel, appendix and cecum affected in 50%-90%, 2%-16%, 3%-18% and 2%-5% of cases, respectively[4]. As in our case, ileocecal involvement is rare with an incidence of 4.1% in intestinal cases[7].

The etiology of endometriosis is still elusive. The most widely accepted theory is that "retrograde menstruation" causes the implantation and growth of endometriosis on the serosal surface of extra-uterine organs or that this occurs secondary to metaplasia in the pelvic peritoneum[2,8-10].

Symptoms of bowel endometriosis can be associated with the patient's menstrual cycle in 18%-40% of cases but may become permanent when the lesions progress[2,11,12]. Under cyclical hormonal influences, serosal implants may proliferate and infiltrate the bowel wall, and lead to inflammation, fibrosis, and metaplasia or hyperplasia of intestinal smooth muscles that can involve the serosa, submucosa and (uncommonly) mucosa[13]. This then leads to introverted mucosa surrounding the appendix hole, luminal stricture, longitudinal ulcer, and ileocecal mass as we believe happened in our case.

Symptoms range from an asymptomatic state to a constellation of symptoms like painful bowel movements, cramps, constipation, diarrhea, vomiting, rectal pain, infertility, abdominal mass, increased urinary frequency and cyclical hematochezia[3]. Those symptoms can mimic a wide spectrum of diseases, including irritable bowel syndrome, infectious diseases, ischemic enteritis/colitis, inflammatory bowel disease and neoplasm, so it is difficult to establish a preoperative diagnosis of bowel endometriosis[2,14,15].

Laboratory tests such as CA125 detection are not sensitive enough for diagnosis[9]. Transvaginal sonography should be used as the first-line diagnostic technique; this has shown a sensitivity and specificity of 43.7% and 50%, respectively[16,17]. Saline contrast sonovaginography was more accurate in diagnosing the condition than was transvaginal ultrasonography, with a sensitivity and specificity of 90.6% and 85.7%, respectively[16-18]. Contrast CT with enteroclysis protocols can be useful in diagnosis as this may demonstrate focal or constricting bowel lesions[4,9]. Magnetic resonance imaging (MRI) is currently the best imaging modality for enteric endometriosis with a sensitivity of 77%-93%[1,9]. Endoscopy may provide no valuable results because of the intact mucosa, but it is still recommended in all patients with suspected endometriosis to rule out mucosal involvement and malignant lesions with the help of biopsies, if needed. In our patient, symptoms relapsed irregularly and were not related with menses. The imaging results showed an ileocecal mass and longitudinal ulcer with luminal stricture under endoscopy, which strongly suggested CD. Due to the bowel

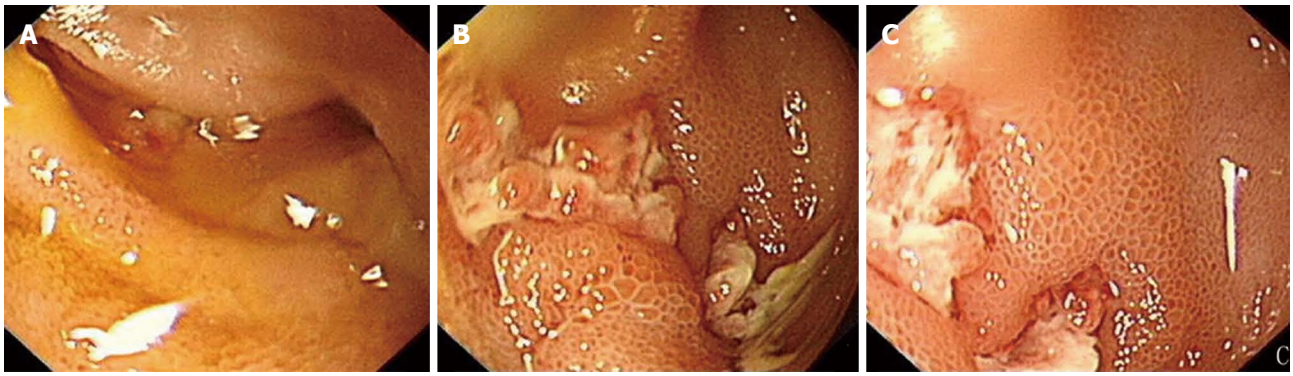

Figure 1 Enteroscopic findings of the patient. A: Mucosal edema of the distal ileum; B: A deep longitudinal ulcer and luminal stricture in distal ileum; C: Multiple ulcers, mucosal edema in distal ileum.

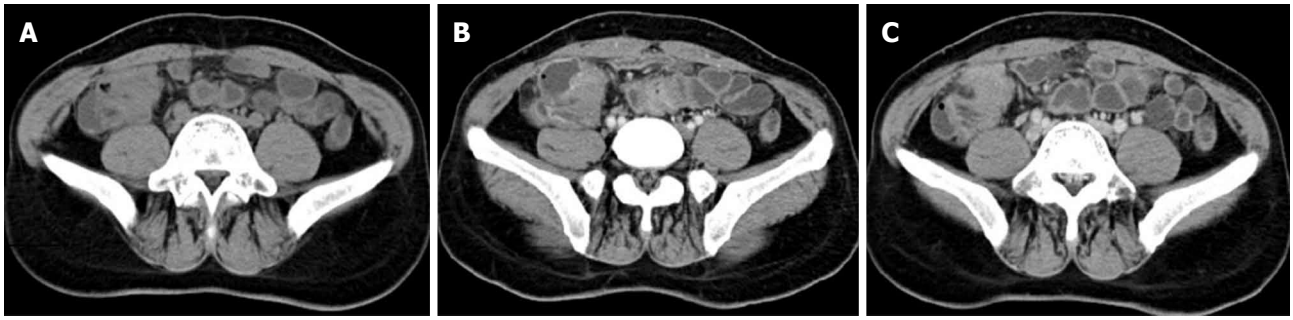

Figure 2 Small bowel computer tomography scan of the patient. Ileocecal bowel wall thickening and luminal narrowing with proximal lumen expansion. Contrast enhancement pattern showed marked enhancement. No other parts of the intestine involved.

obstruction, surgery was recommended.

Histopathological confirmation required presence of both glandular and stromal tissue. In our patient, the pathologist's findings showed that the annular lesion of endometriosis and mucosa was not involved.

The treatment of uncomplicated intestinal endometriosis depends on the patient's age and intention to conceive. Medical treatment with hormonal therapy such as the oral contraceptive pill, danazol or gonadotropin antagonists can be attempted for intestinal disease when there is no obstruction[1,2,19]. Bowel resection is indicated if there are symptoms of obstruction or bleeding, and if malignancy cannot be excluded. Post-operative hormonal therapy does not demonstrate benefits, according to a recent meta-analysis[20].

In summary, bowel endometriosis should be borne in mind when a woman of reproductive age presents with episodic gastrointestinal symptoms. A careful history may elicit symptoms related to the patient's menses. Small bowel CT and MRI is indicated, and endoscopy is still recommended in all patients to rule out mucosal involvement and malignant lesions. In our case, the final diagnosis could only be given by the pathologist's report. Multidisciplinary care should be encouraged to ensure correct evaluation and improve the management of these patients.

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Response to Abadi and Kusters, *World J Gastroenterol* 19: 429-430

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Abstract

In a recent study, Rafiei *et al*, reported a link between a C150T polymorphism in the human inducible nitric oxide gene and *Helicobacter pylori* infection as a risk factor for gastric cancer among an Iranian population. Subsequently, Abadi and Kusters published a letter to the editor questioning the validity of the study because of a supposed flaw in primer design. Here we respond to the claims of Abadi and Kusters and show that the results reported in the original article are valid.

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Key words: Inducible nitric oxide synthetase; *Helicobacter pylori*; Gastric cancer

Core tip: In a recent Letter to the Editor, Abadi and Kusters brought into question the validity of a study published by Rafiei *et al.* Herein we respond to the claims made by Abadi and Kusters, and show that the results reported in the article originally published by Rafiei *et al*, are valid.

Rafiei A, Gilbreath JJ, Merrell DS. Response to Abadi and Kusters, *World J Gastroenterol* 19: 429-430. *World J Gastroenterol* 2013; 19(23): 3711-3712 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i23/3711.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i23.3711

TO THE EDITOR

In a recent letter to the editor, entitled "Association of inducible nitric oxide synthetase genotype and *Helicobacter pylori* infection gastric cancer risk may be due to faulty primer design"[1], Amin Talebi Bezmin Abadi and Johannes Kusters questioned the validity of the results published in our manuscript[2], the design of which was based on a previous study[3]. In their letter[1], the authors appraised our article[2] by two main points; variation in T allele frequency and primer design. In response to their first comment regarding the T allele frequency seen in our population, we stand by the conclusion that genetic polymorphisms depend on ethnic differences; the frequency of mutant genotypes varies in different human populations. Therefore, it is likely no surprise that the frequency of the T allele seen in our Caucasian population[2] differed from that of the East Asian population[3].

In regards to the second point made by Abadi *et al*[1], concerning the primer specificity, we note that there is a misprint in one primer sequence in our article[2]. The primer sequence published in our paper (5'-GTCTCTG-CGGGTCTGAAG-3') differs from that of Shen *et al*[3] in that it is missing two base pairs in the 3' end of the sequence. An erratum has been submitted to the journal to note the misprinted primer sequence [On page 4919[2], the primer sequence given for iNOS-R (5'-174 GTCTCTGCGGGTCTGAAG-3') is missing two nucleotides and should appear as iNOS-R (5'-GTCTCTGC-GGGTCTGAGAAG-3')]. However, this 2 base misprint appears not to be the core issue since the correct primer sequence (5'-GTCTCTGCGGGTCTGAGAAG-3') was

GCCCCATA***TGTAAACCAACTTCCGTGGTG***GGCTGTGAGCCTTCTCCTGCAAGCTGTGGCCAGGTTTCC
AG AAGAAAGGAAAAAACAGTGTTTTTATCATCCTGGCTTGAGAACTCTGTCATTCCCTCTCTTTCTAGAAAC
TGAAGAAAT**C**GCTCTTCATGCTGAAAGAGCTCAACAACAAATTCAGGTAAAGCTTTCTCCGGTGTCCTAC
TCCTAGCCCTGCCCTGGGGCCCCAGTGTCTGGTCACAGGGGATAGCTCTGGGTGTACACAGGGGGT***CTTC***
TCAGACCCGCAGAGACACAGAGC

Figure 1 Location of inducible nitric oxide synthetase-specific primer annealing sites. The sequence of exon 16 of the inducible nitric oxide synthetase (*iNOS*) gene (http://www.ncbi.nlm.nih.gov/nuccore/x85772) is shown. The sequence of forward primer used in our study and by Shen *et al*[3] is in bold italics at the 5' end of the sequence above: 5'-TGTAAACCAACTTCCGTGGTG-3'. The sequence of the reverse primer used in our study and by Shen *et al*[3] (5'-GTCTCTGCGGGTCT-GAGAAG-3') is the reverse compliment of the bold italicized bases in the 3' end of the sequence above. The C150T polymorphism is indicated by the bold underlined "C".

previously published by Shen *et al*[3] and this publication was also called into question by Abadi and Kusters. We note that as shown in Figure 1, both of the primers used in the study are specific to exon 16 of the *iNOS* gene (Figure 1), which has been mapped to chromosome 17q11.2. Direct sequence analysis of exon 16 as submitted to GenBank by Shen *et al*[3] (http://www.ncbi.nlm.nih.gov/nuccore/x85772) shows that the primers used in our studies are completely conserved in this sequence. Furthermore, blast analysis of the inducible nitric oxidase synthase against the human database returns 3 hits to Homo sapiens chromosome 17. Analysis of the resulting alignments shows complete conservation of the forward primer in all 3 samples. Conservation of the reverse primer is not as high; the 5' end of the primer shows several mismatches. However, the last 9 nucleotides found in the 3' end of the primer are completely conserved across all 3 samples. Thus, there should be no overt obstacles to amplification of the gene in question. In conclusion, we stand by the results reported in our original study[2] and posit that the supposition by Abadi and Kusters that our study design is flawed is incorrect.

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Acknowledgments

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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

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